

**Interactions of foot-and-mouth disease virus with
cells in organised lymphoid tissue influence innate
and adaptive immune responses**

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Declaration

I hereby declare that the research described within this thesis is my own work, unless acknowledged in the text. I certify that the work has not been submitted for any other degree or professional qualification.

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Abstract

Foot-and-mouth disease virus (FMDV) is one of the most contagious viruses of animals and is recognised as the most important constraint to international trade in animals and animal products. Two fundamental problems remain to be understood before more effective control measures can be put in place. These problems are the FMDV 'carrier state' and the short duration of immunity after vaccination which contrasts with prolonged immunity after natural infection. The aim of this thesis was to study the interaction between FMDV and cells in lymphoid tissue in the natural bovine host, in order to improve our understanding of the protective immune response.

Using laser capture microdissection in combination with quantitative real-time reverse transcription polymerase chain reaction, immunohistochemical analysis and corroborated by *in situ* hybridization, it is shown that FMDV locates rapidly to, and is maintained in, the light zone of germinal centres following primary infection of naïve cattle. Maintenance of non-replicating FMDV in these sites may represent a source of persisting infectious virus and also contribute to the generation of long-lasting antibody responses against neutralising epitopes of the virus.

The role of T-lymphocyte subsets in recovery from FMDV infection in calves was investigated by administering subset-specific mouse monoclonal antibodies.

Depletion of circulating CD4⁺ or WC1⁺ $\gamma\delta$ T cells was achieved for a period extending from before challenge to after resolution of viraemia and peak clinical signs, whereas CD8⁺ cell depletion was only partial. Depletion of CD4⁺ cells was

also confirmed by analysis of lymph node biopsies 5 days post-challenge. Depletion with anti-WC1 and anti-CD8 antibodies had no effect on the kinetics of infection, clinical signs and immune responses following FMDV infection. Three of the four CD4⁺ T-cell-depleted calves failed to generate an antibody response to the non-structural polyprotein 3ABC, but generated a neutralising antibody response similar to that in the controls, including rapid isotype switching to IgG antibody. These data suggest that antibody responses to sites on the surface of the virus capsid are T cell-independent whereas those directed against the non-structural proteins are T cell-dependent. CD4 depletion was found to substantially inhibit antibody responses to the G-H peptide loop VP1₁₃₅₋₁₅₆ on the viral capsid, indicating that responses to this particular site, which has a more mobile structure than other neutralising sites on the virus capsid, are T cell-dependent. Depletion of CD4⁺ T cells had no adverse effect on the magnitude or duration of clinical signs or clearance of virus from the circulation. In conclusion, CD4⁺ T-cell-independent antibody responses play a major role in the resolution of primary infection with FMDV in cattle.

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List of Abbreviations

ANOVA	Analysis of variance
APRIL	A proliferation activation ligand of the TNF family
BAFF	B-cell activating factor of the TNF family
BCMA	B-cell maturation antigen
BCR	B-cell antigen receptor
BHK	Baby hamster kidney
BLN	Bronchial lymph node
BTY	Bovine thyroid
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CDR	Complementary-determining region
Cre	Cis-acting replication element
CSU	Central services unit
Ct	Threshold cycle
DAPI	4'-6-Diamidino-2-phenylindole
DC	Dendritic cells
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
DSP	Dorsal soft palate
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
Fc	Fragment crystallisable
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
FSC	Forward scatter
GC	Germinal centre
GMEM	Glasgows Modified Eagle's Medium
H&E	Hematoxylin and eosin
HEV	High endothelial venules
HIV	Human immunodeficiency virus
IAH	Institute for Animal Health
ICAM	Inter-cellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRES	Internal ribosomal entry site
LCM	Laser capture microdissection
LCMV	Lymphocytic choriomeningitis virus
L ^{pro}	Leader protease
LT	Lymphotoxin
MΦ	Monocyte derived macrophage

MAB	Monoclonal antibody
MAdCAM	Mucosal vascular addressin cell adhesion molecule
MALT	Mucosal associated lymphoid tissue
MHC	Major histocompatibility complex
MIF	Macrophage migration-inhibitory factor
MLN	Mandibular lymph node
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NK	Natural killer
NOG	n-octyl- β -d-glucopyranoside
OD	Optical density
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	plasmacytoid dendritic cell
Poly (C)	Polyribocytidylate
RGD	Arginine-glycine-aspartate
RNA	Ribonucleic acid
RPLN	Lateral retropharyngeal lymph node
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal ribonucleic acid
rRT-PCR	Real time reverse transcription polymerase chain reaction
SAT	Southern African territories
SCID	Severe combined immunodeficiency
SNT	Serum neutralising antibody titre
SSC	Side scatter
SVD	Swine vesicular disease
SVDV	Swine vesicular disease virus
TCID	Tissue culture infectious dose
TCR	T-cell receptor
T-D	T-dependent
T-I	T-independent
TLR	Toll-like receptor
TSA	Tyramide signal amplification
TMEV	Theiler's murine encephalomyelitis virus
TNF	Tumour necrosis factor
UTP	Uracil triphosphate
UTR	Untranslated region
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VLP	virus-like particle
VSV	Vesicular stomatitis virus
WC	Workshop cluster

1. General introduction

The livestock sector plays a vital role in the economies of many developing countries by providing food, income, a means of transport, draught power and employment (Otte *et al.*, 2004). An estimated 600 million people worldwide rely directly on livestock production for their livelihoods. In addition, the population of developing countries grows by an estimated 72 million each year and the average meat consumption in the developed world is increasing, adding to the demand for meat products (Caspari, 2007). Livestock diseases constitute a major barrier to agricultural and economic development. Transboundary animal diseases pose the biggest threat to the livestock industry. Transboundary animal diseases are defined as “those that are of significant economic, trade and/or food security importance for a considerable number of countries; which can easily spread to other countries and reach epidemic proportions; and where control/management, including exclusion, requires cooperation between several countries” (Otte *et al.*, 2004). Significant transboundary animal diseases identified by the Food and Agriculture Organisation include rinderpest, contagious bovine pleuropneumonia, bovine spongiform encephalopathy, rift valley fever, peste des petits ruminants, classical swine fever, African swine fever, Newcastle disease and of particular importance; avian influenza and foot-and-mouth disease (FMD) (Caspari, 2007).

Although FMD is not important from a public health perspective, it significantly constrains smallholder livestock producers and has a significant socio-economic impact in the developing and the developed world (Perry and Rich, 2007).

Subsequently, the prevention, control and eradication of FMD attracts a significant amount of effort and resources.

1.1. Foot-and-mouth disease

FMD is a highly contagious, acute vesicular disease, caused by FMD virus (FMDV), which affects wild and domestic cloven-hoofed animals (Alexandersen *et al.*, 2003b). It is endemic in many areas of Asia, Africa, South America and eastern Europe where it plays an important role in the limitation of international trade of livestock and livestock products and impacts the livelihood of the poor (Perry and Rich, 2007). The ability of the virus to infect in small doses, multiple modes of infection and wide host range make FMD a difficult and expensive disease to control and the cost of eradication can be enormous (Scudamore, 2002). The achievement and maintenance of FMD-free status has major benefits for international trade and countries free of disease take great precautions to maintain their disease-free status. Cost-benefit analyses have indicated that the potential economic benefits of FMD control in many endemic situations outweighs the financial investment associated with eradication (Caspari, 2007).

FMD can be established in susceptible animals by direct or indirect contact with infected animals, inhalation of airborne virus or contact with contaminated animal products, materials and people (Alexandersen *et al.*, 2003b). The length of the incubation period is highly variable under field conditions and dependent on the infecting strain, the dose and route of infection, the animal species, individual susceptibility and the husbandry and environmental conditions (Quan *et al.*, 2004).

The reported incubation period for farm-to-farm and within-farm spread is between 1 to 14 days (Garland and Donaldson, 1990, Sellers and Forman, 1973). The length of the incubation period under experimental conditions is also variable and influenced by the same factors for field conditions. The reported mean incubation periods under experimental conditions are 3.5 days, 2 days and 1 to 3 days for cattle, sheep and pigs respectively (Alexandersen *et al.*, 2003a).

The literature provides evidence that transmission of FMDV in domestic animals by the nasal or oral route results in primary infection and replication in the dorsal soft palate and the roof of the pharynx. The stratified squamous respiratory epithelium and tonsils in these areas are thought to be important for primary replication of the virus (Alexandersen *et al.*, 2003b, Prato Murphy *et al.*, 1999). Following aerosol inoculation of FMDV in cattle, virus can also be detected in the lung (Pacheco *et al.*, 2008). However, it is still unclear what part lung tissue plays in primary infection as a site of entry or secondary viral amplification (Alexandersen *et al.*, 2003b). Authors of *in situ* hybridization studies suggested that early replication takes place in lung tissue and alveolar macrophages after aerosol exposure with subsequent dissemination to distal sites (Brown *et al.*, 1992, Brown *et al.*, 1996).

Following primary replication, the virus disseminates rapidly through the host. Dissemination of the virus from the primary sites of infection to the rest of the body is thought to occur via the lymphatic and circulatory system, however, the mode of dissemination still remains inconclusive. There is no significant evidence for replication or transport of FMDV in bovine peripheral blood mononuclear cells

(PBMC) (Zhang and Alexandersen, 2004). However, a transient lymphopenia has been noted during the early stages of infection in swine (Bautista *et al.*, 2003). The susceptibility of porcine PBMC to active infection during the acute stages of FMD may depend on the serotype of virus. PBMC isolated from serotype C infected swine were shown to be actively infected with viral titres corresponding to the period of peak viraemia as determined by infectious centre assays (Bautista *et al.*, 2003). In contrast, PBMC isolated from serotype O infected swine during the acute stages of FMD were not infected (Diaz-San Segundo *et al.*, 2006). Macrophages and Langerhans cells are considered to take part in virus dissemination (Brown *et al.*, 1992, Brown *et al.*, 1995, David *et al.*, 1995, di Girolamo *et al.*, 1985, Summerfield *et al.*, 2008), however, more data is required to determine the ability of bovine immune cells to support virus replication and transport. The greater part of viral amplification is considered to occur within the cornified stratified squamous epithelium of the skin, particularly in and around the mouth, feet and mammary glands, distinguishing these tissues as the sites of secondary replication (Alexandersen *et al.*, 2003b). Interestingly, in calves exposed to aerosol virus, FMDV RNA was detected in coronary band and interdigital epithelium as early as six hours post-infection, before the onset of viraemia and clinical signs (Brown *et al.*, 1992).

FMD in livestock is characterised by high morbidity and low mortality in adult animals. The earliest clinical signs in cattle include pyrexia, depression, a fall in milk yield and cessation of rumination. These signs are superseded within a few hours by vesicles at the sites of secondary replication, which are characteristic of FMD. FMD

vesicles generally rupture within 1 to 2 days resulting in the formation of erosions. Erosions on the feet cause lameness and are often complicated by secondary bacterial infections which delay the healing process. Although mortality is rare in adults, infection can cause abortion and the virus can replicate in the myocardium of lambs which can be fatal (Gulbahar *et al.*, 2007).

The viraemic phase in cattle lasts approximately for 3 to 5 days and FMDV is normally cleared from peripheral sites within 2 weeks (Salt, 2004, Zhang and Alexandersen, 2004). However, FMDV can cause a prolonged, asymptomatic infection in ruminants leading to the ‘carrier’ state. ‘Carriers’ are defined as animals from which live-virus can be recovered from scrapings of the oropharynx, using a probang sampling cup, after 28 days following infection (Sutmoller and Gaggero, 1965). The oropharynx and dorsal soft palate have been implicated as the sites of viral persistence (Burrows, 1966), yet the cell type maintaining FMDV during persistence *in vivo* has not been conclusively identified and no confirmed mechanism of persistence has been reported.

1.2. Foot-and-mouth disease virus

1.2.1. Classification and structure

FMDV is a member of the family *Picornaviridae* which consists of 8 genera, *Enterovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Kobuvirus* and *Teschovirus*. The genus *Aphthovirus* consists of two species, FMDV and Equine rhinitis A. Equine rhinitis A virus, which is closely related to FMDV, causes a respiratory infection in horses characterised by coughing, anorexia,

pharyngitis and persistent virus shedding from the pharyngeal region and in the urine and faeces (Kriegshäuser *et al.*, 2005). Recent nucleotide sequence data has shown that bovine rhinoviruses, which are associated with respiratory disease in cattle, should be re-classified in the genus *Aphthovirus* (Hollister *et al.*, 2008).

The first reference to FMD is that by Hieronymus Fracastorius, who described the disease in cattle in Italy in 1514 (Fracastorius, 1546). During the latter half of the 19th century, FMDV was identified as the first animal agent to cause disease that was small enough to pass through Berkfeld filters, and only the second virus to be discovered (Loeffler and Frosch, 1898). It was soon observed that cattle which had recovered from FMD were resistant to re-infection, however this was not always the case and serotypes were assigned on the basis of lack of cross protection. The serotype prevalent at this time in France was designated type O as it originated from the Oise valley. The virus that re-infected type O recovered animals was called type A, for “Allemagne”, as it originated from Germany (Vallée and Carré, 1922). A third serotype was discovered soon afterwards, designated C as the authors wanted to rename the serotypes A, B and C (Waldmann and Trautwein, 1926). The Southern African Territories (SAT) 1, 2 and 3 serotypes were described by the Pirbright laboratory in 1948 (Brooksby, 1958) and the final serotype, Asia 1 was typed from a sample from Pakistan in 1954 (Brooksby and Rogers, 1957). Based on genome analysis (Figure 1), types O, A, C and Asia 1 constitute a clear evolutionary lineage distinguishable from the SAT serotypes (Knowles and Samuel, 2003). Most human and animal RNA viruses display extensive genetic and antigenic heterogeneity

within infected hosts and populations, FMDV is no exception and within a serotype wide ranges of subtypes occur (Domingo *et al.*, 2002, Hernandez *et al.*, 1992).

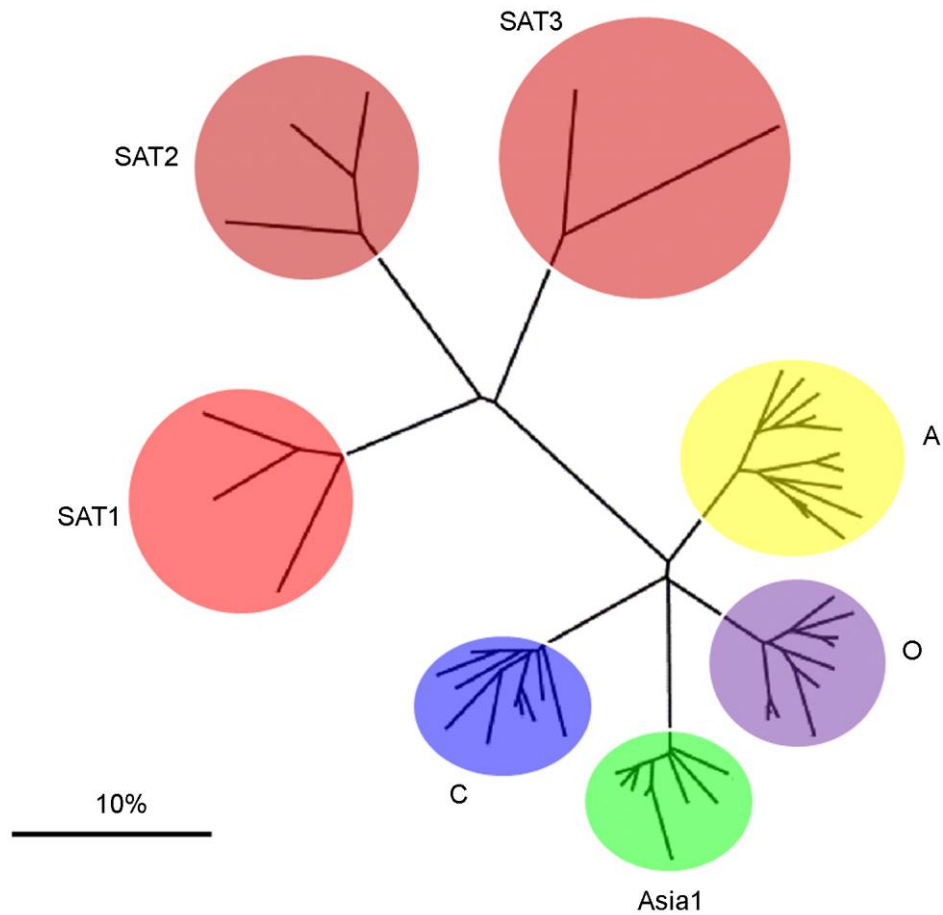


Figure 1. Unrooted Neighbour-joining tree showing the relationships between the outer-capsid polypeptides of FMDV.

Unrooted Neighbour-joining tree shows the relationship between the outer-capsid polypeptides (VP1, VP2 and VP3) of the seven FMDV serotypes (O, A, C, Asia 1, SAT1 to 3). The seven FMDV serotypes cluster into type-specific lineages when comparing either nucleotide or amino acid sequences. Adapted from Knowles and Samuel, 2002.

The FMDV particle consists of a non-enveloped icosahedral protein shell (capsid) contained a single stranded positive sense RNA genome approximately 8500 nucleotides in length (Forss *et al.*, 1984). The capsid is comprised of 60 copies each of the four structural proteins VP1 (1D), VP2 (1B), VP3 (1C), and VP4 (1A). These four proteins assemble to form a protomer and five protomers join to form a pentamer. Twelve pentamers join to enclose the genomic RNA creating the virus particle (Acharya *et al.*, 1989). VP1 to 3 are surface orientated, while VP4 is internal and in contact with the RNA (Figure 2). The surface structural proteins VP1 to 3 of FMDV are smaller than their counterparts in other picornaviruses. In addition, FMDV lacks distinctive surface features such as canyons and pits which have been described for other picornaviruses (Acharya *et al.*, 1989, Hogle *et al.*, 1985, Parry *et al.*, 1990). It has been suggested that the canyons and pits protect the site of cell receptor attachment from the humoral immune response, in addition, receptor-binding into the canyon destabilises the virus to initiate the uncoating process (Rossmann *et al.*, 2002). In contrast, a long protein loop containing elements of the cell attachment site and the major viral antigenic site of FMDV forms a highly accessible protrusion which distinguishes FMDV from other picornaviruses (Acharya *et al.*, 1989). Crystallographic studies of the three-dimensional structure of several FMDV isolates and antigenic variants have been reported, these studies have shown that VP1 to 3 have the same eight-stranded β -barrel folding motif (Figure 2) seen in other picornaviruses (Acharya *et al.*, 1989, Curry *et al.*, 1996, Logan *et al.*, 1993, Parry *et al.*, 1990). Protein loops, joining the β -strands and C-termini of the surface structural proteins are exposed on the surface of the capsid (Figure 2). The highly exposed and flexible G-H loop, also called the “FMDV loop”, of VP1

contains an antigenic site and the conserved sequence arginine-glycine-aspartate (RGD) which constitutes the main cellular attachment site for integrin recognition (Logan *et al.*, 1993).

Antibodies are considered as the major effector for protection against FMD, therefore a number of studies have focused on the structural and functional aspects of their interaction with FMDV. Crystallographic studies of serotypes O, A and C have shown that major conformational differences and sequence variability between the capsid proteins of these serotypes exists in their loop structures and C-terminal segments, and these regions define their antigenic character (Acharya *et al.*, 1989, Curry *et al.*, 1996, Lea *et al.*, 1995, Lea *et al.*, 1994). Multiple antigenic sites have been described for FMDV. A site is defined as a discrete area on the antigen surface where a B-cell epitope or several overlapping epitopes have been mapped by monoclonal antibodies (MAbs) (Mateu and Verdaguer, 2004). The B-cell epitope denotes the part of the antigen recognised by a specific antibody. These epitopes are defined as ‘continuous’ or ‘linear’ when they are contained within a short peptide sequence, for example, a single loop of a folded protein, or as ‘discontinuous’ or ‘conformational’ when they are formed by residues that are located apart in the primary structure, but are brought together in the folded protein conformation (Mateu, 1995).

Cross neutralisation assays and sequencing of different FMDV serotype O MAb resistant mutants has identified 5 antigenic sites (Figure 2) on the virus particle involved in virus neutralisation, these sites are often referred to in the literature as the

“major antigenic sites” (McCullough *et al.*, 1987a). Site 1 involves both the trypsin-sensitive residues in the G-H loop (site 1a) and the VP1 C-terminus (site 1b), because mutations that allow escape from the same MAb were described in either region (Kitson *et al.*, 1990, Strohmaier *et al.*, 1982). Site 2 involves residues within the two surface loops B-C and E-F of VP2 (Kitson *et al.*, 1990, Mateu and Verdaguer, 2004). Sites 3 and 4 involve residues within the B-C loop of VP1 and B-B knob of VP3 respectively (Kitson *et al.*, 1990) . A fifth functionally independent site is located within the G-H loop of VP1 (Crowther *et al.*, 1993).

FMDV is insensitive to organic solvents, as the virus lacks a lipid envelope, however the virus particles are unstable at pH below 6.8. In common with other picornaviruses, heat or acid degradation causes the capsid to dissociate into its pentameric subunits and VP4 forms an insoluble aggregate, releasing the RNA (Brown and Cartwright, 1961). The FMDV RNA genome can be divided into three main functional regions, the 5' untranslated region (UTR), the protein coding region consisting of a single open reading frame (ORF) and the 3' UTR (Figure 3). The FMDV genome is infectious and no viral proteins are required to initiate replication, a feature consistent with other picornavirus RNA (Belsham and Bostock, 1988).

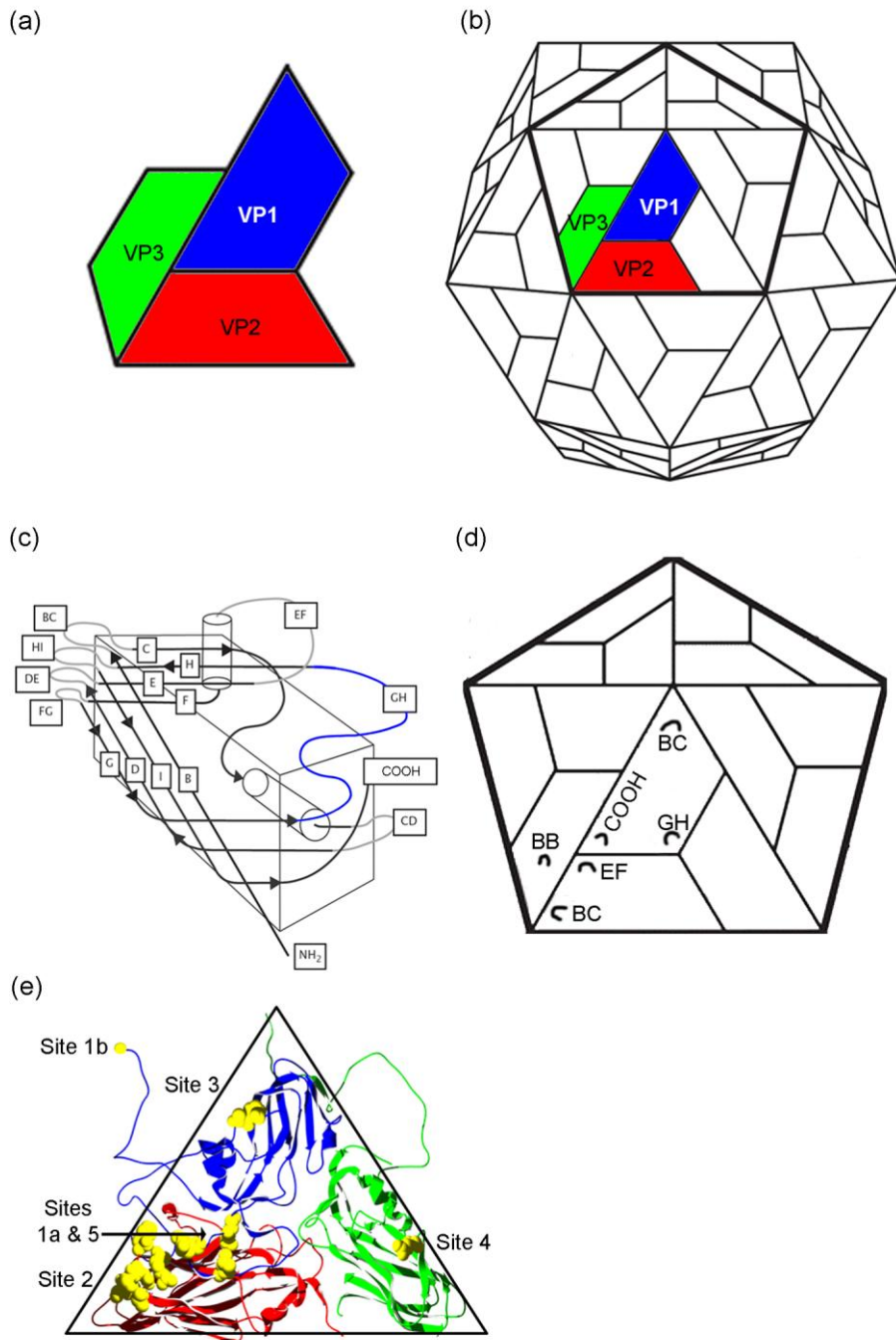


Figure 2. Structure of foot-and-mouth disease virus particles.

(a) Arrangement of the three surface proteins VP1 (blue), VP2 (red) and VP3 (green) in a protomer. (b) Structure of the capsid. A pentamer, consisting of 5 protomers arrayed in five-fold rotational symmetry about the pentagonal centre, is outlined in the capsid and a protomer is indicated inside the pentamer. Each protein presents an approximately trapezoidal shape on the surface. Adapted from Sobrino *et al.*, 2001. (c) Topology of the wedge-shaped eight-stranded β -barrel fold found in icosahedral, positive-strand RNA viruses (Harrison, 1989). Eight β chains (arrows) labelled B to I and two α chains (cylinders). The loops connecting the β chains tend to be exposed on the protein surface (G-H loop of VP1 highlighted in blue), sometimes protruding

from the protein core. The two-letter codes for the loops name the connected β chains. The carboxyl (COOH) and amino (NH₂) termini may also occur at the surface. Adapted from Frank, 2002. (d) A pentamer viewed from above. Lines labelled on one protomer represent the location of 5 antigenic sites on the virus particle involved in virus neutralisation. The sites were identified by cross neutralisation assays and sequencing of different FMDV serotype O MAb resistant mutants. These sites are often referred to in the literature as the “major antigenic sites” (McCullough et al., 1987a). Site 1 involves both the trypsin-sensitive residues in the G-H loop (GH, site 1a) and the VP1 C-terminus (COOH, site 1b), because mutations that allow escape from the same MAb were described in either region (Kitson et al., 1990, Strohmaier et al., 1982). Site 2 involves residues within the two surface loops B-C (BC) and E-F (EF) of VP2 (Kitson et al., 1990, Mateu and Verdaguer, 2004). Sites 3 and 4 involve residues within the B-C (BC) loop of VP1 and B-B (BB) knob of VP3 respectively (Kitson et al., 1990). A fifth functionally independent site is located within the G-H (GH) loop of VP1 (Crowther et al., 1993). Adapted from Frank, 2002. (e) Ribbon representation of VP1 (blue), VP2 (red) and VP3 (green). Locations of the 5 antigenic sites are shown in yellow. Adapted from Belsham *et al.*, 2008.

1.2.1.1. The FMDV 5' UTR

The 5' UTR of FMDV is larger than the UTR of most other picornaviruses and can be considered to be composed of various regions including the S-fragment, a polyribocytidylate [poly (C)] tract, the *cis*-acting replication element (*cre*) and the internal ribosomal entry site (IRES) (Biswas *et al.*, 2005). The function of the S-fragment, which is approximately 360 nucleotides in length, has not been characterised, however it may serve to circularise the RNA and may facilitate replication and/or translation (Herold and Andino, 2001). The S-fragment is followed by the poly(C) tract, which varies in length amongst different strains of FMDV but the significance of the size of this sequence is not clear (Mellor *et al.*, 1985). Upstream from the *cre* are multiple pseudoknots that may be involved in a joint function with the poly(C) tract (Belsham and Martinez-Salas, 2004). The *cre* is a stable stem loop structure upstream of the IRES in FMDV that is essential for replication of the picornavirus RNA (Mason *et al.*, 2002, Tiley *et al.*, 2003). The FMDV IRES is a highly structured region of approximately 450 nucleotides that serves for the internal initiation of viral protein synthesis in a cap-independent fashion (Roberts *et al.*, 1998). In contrast, eukaryotic mRNA translation depends on the recognition of the 7-methyl-G cap structure at the 5' end of the mRNA and the heterotrimeric initiation factor eIF4F composed of eIF4E, eIF3 and eIF4G which interacts with the small ribosomal subunit (Gingras *et al.*, 1999).

1.2.1.2. The ORF

The ORF, a region of approximately 7000 nucleotides, encodes a polyprotein, the full length polyprotein is never detected in infected cells or during *in vitro* translation

reactions since primary processing of the nascent polypeptide begins co-translationally (Belsham and Martinez-Salas, 2004). The viral proteins are generated from the polyprotein through the cleavage activities of two-*trans* acting virus encoded proteases, namely the L protease (L^{pro}) and 3C protease, and by 2A protein (Belsham *et al.*, 2008). The L^{pro} cleaves itself from the viral polyprotein at the L/P1 junction (Figure 3), releasing the P1-2A precursor at its N-terminus (Belsham, 2005). The P1-2A capsid precursor is released at the junction between the C-terminus of the short 2A peptide and the N-terminus of the 2B region, a process mediated by the 2A sequence together with the first amino acid of 2B (Ryan *et al.*, 1991). It has been proposed that this event is not in fact a proteolytic cleavage of an existing peptide bond, but instead results from a modification of translation such that the bond is never formed but translation of the downstream sequence still continues (Donnelly *et al.*, 2001). The properties of the 2A oligopeptide together with the first residue of 2B (a proline) can also mediate cleavage in artificial polyprotein systems (Donnelly *et al.*, 1997). The P1-2A capsid precursor is processed further by 3C protease to yield VP0 (1AB, which is the precursor for VP4 and VP2), VP3 (1C) and VP1 (1D) (Belsham, 2005). The P2 precursor is processed into 2B and 2C by 3C protease. Although the function of these proteins and precursors is not entirely clear, they have been shown to enhance membrane permeability and may assist in evasion of the host immune response by blocking protein secretory pathways (Belsham, 2005, Moffat *et al.*, 2005). The L^{pro} mediates cleavage of eIF4G, FMDV 3C protease also takes part in shutting off host cap-dependent mRNA translation by cleaving eIF4A and eIF4G, although this cleavage occurs later in the infection cycle (Belsham, 2005, Belsham *et al.*, 2000)

The FMDV P3 precursor is processed by the 3C protease into 3A, three copies of the 3B peptide (VPg), 3C protease and 3D polymerase, in addition, a variety of intermediates are produced during processing (Figure 3) (Vakharia *et al.*, 1987). The 3A protein serves to localise the FMDV RNA to membrane vesicles (Rosas *et al.*, 2008) and is thought to deliver 3B peptides, which act as primers for RNA synthesis, to the sites of RNA replication (Nayak *et al.*, 2005, O'Donnell *et al.*, 2001). The 3D polymerase is thought to recognise both positive and negative sense viral RNA.

1.2.1.3. The FMDV 3' UTR

The 3'UTR is composed of a heterogeneous sequence and the poly(A) tail. Information about the role of these different regions is limited. The heterogeneous sequence has been shown to stimulate IRES activity (Lopez de Quinto *et al.*, 2002) and is crucial for virus infectivity (Saiz *et al.*, 2001). The poly(A) tract, which unlike cellular mRNA, is encoded by the genome, may be important for RNA stability and for a possible interaction between the 3' and 5' UTR.

1.2.1.4. Synthesis of viral RNA

The FMDV genomic RNA functions both as mRNA to produce virus-encoded proteins and as a template for the production of new RNA transcripts (Nayak *et al.*, 2005). Translation of the viral RNA must precede RNA replication so that viral proteins required for replication are generated within the infected cell. At some point there has to be a switch in the function of the input genomic RNA so that translation is blocked and RNA synthesis can commence. This is required because the process

of translation in which the ribosomes move along the RNA in a 5' to 3' direction is not compatible with the movement of the 3D polymerase in the 3' to 5' direction (Belsham and Martinez-Salas, 2004, Gamarnik and Andino, 1998). The genomic RNA is uncapped but is linked at its 5' end to the virus encoded peptide VPg (Nayak *et al.*, 2006). The primer for initiating RNA synthesis is the peptide VPg or its precursor, 3AB (Belsham *et al.*, 2008). FMDV makes three alternative forms of VPg which are incorporated at the 5' end of new RNA strands at equal frequencies (Belsham *et al.*, 2008). The uridylylation of the VPg peptide primer is the first stage in the replication of picornavirus RNA (Nayak *et al.*, 2006). The VPg is modified by the addition of uridyl residues to produce VPgpUpU in a reaction involving 3D polymerase, its precursor 3CD and the *cre* (Belsham *et al.*, 2008). Attachment of this peptide to the RNA occurs via a Tyr residue and is performed by the 3D polymerase. RNA synthesis by the virus encoded RNA-dependent 3D polymerase takes place within membrane-bound replication complexes in a two-stage process, the genomic RNA is used to make an antisense copy, the antisense copy is then used as a template for the production of new genomic RNA. The genomic RNA can then be translated to make more viral protein, it can also be packaged into new virus particles or it can be used as a template for making more antisense template. Considerably more genomic RNA molecules are made than the antisense template (Belsham *et al.*, 2008). It is not clear how the genomic RNA molecules are packaged into virions, empty capsid formation can occur in the absence of virion RNA however it is not clear if this is a dead-end product or to what extent the capsid proteins assemble prior to virion assembly (Belsham, 2005).

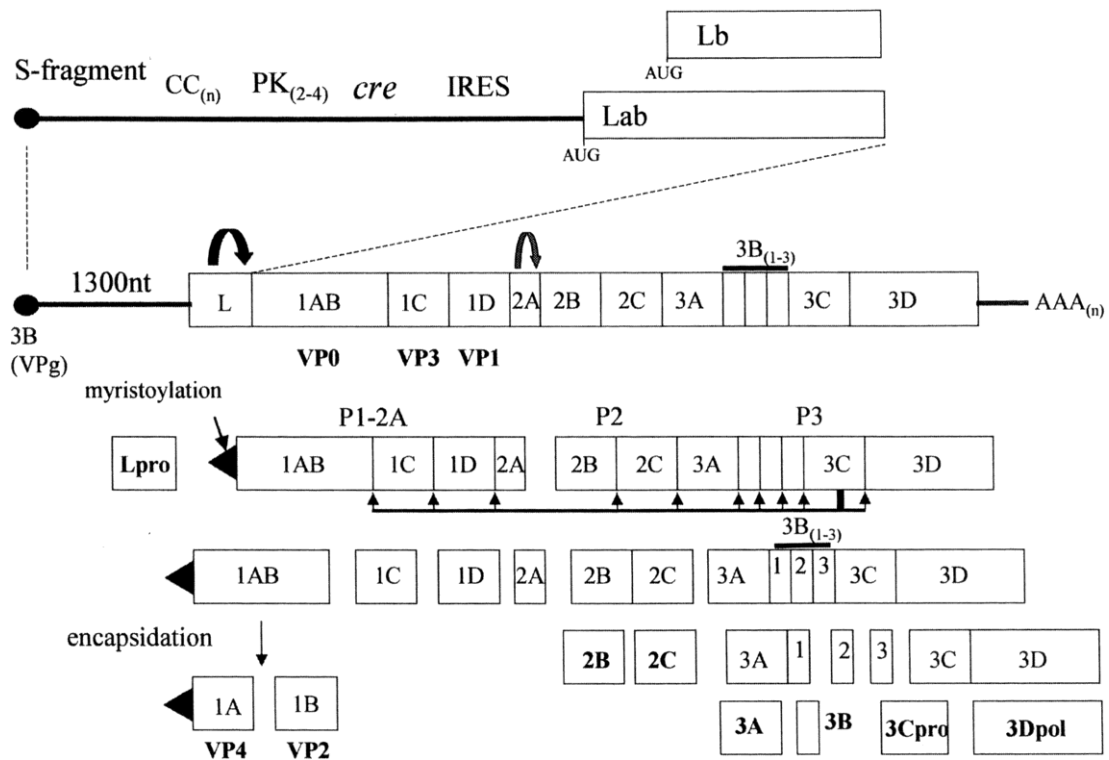


Figure 3. Genome organisation of FMDV.

Genome organisation and polyprotein processing of FMDV (reproduced from Belsham and Martinez-Salas, 2004). The FMDV genome can be divided into three main functional regions, the 5' UTR, a single ORF that encodes a polyprotein which is cleaved by viral proteases into the products indicated and a 3' UTR with a poly(A) tail (AAA_(n)). The 5' UTR is composed of various regions including the S-fragment, a poly(C) tract (CC_(n)), the *cis*-acting replication element (*cre*) and the internal ribosomal entry site (IRES). Upstream from the *cre* are multiple pseudoknots (PK₍₂₋₄₎) that may be involved in a joint function with the poly(C) tract. At the 3' end of the IRES element a polypyrimidine tract is followed by both AUG codons approximately 84nt apart. Both AUG codons are used as initiation sites for protein synthesis and thus 2 distinct forms of the Leader (L) protein are generated termed the Lb and Lab which differ in their N-termini. The sites of primary cleavage and the virus proteins responsible are indicated by the curved arrows. The FMDV polyprotein undergoes primary cleavage at the L/1A junction and the C-terminus of protein 2A. Secondary processing of the primary cleavage products gives rise to a series of alternative products as described under section 1.2.1.2. The viral RNA is synthesised by the virus encoded RNA-dependent RNA polymerase (3Dpol), the viral protein VPg (3B) acts as the primer for RNA synthesis.

1.2.2. Cell entry and replication

FMDV initiates infection of cells by attaching to the host cell membrane by surface receptors. Two classes of receptors have been recognised for FMDV, integrins and heparin sulphate proteoglycans (Jackson *et al.*, 1996). Four RGD-dependent integrins, $\alpha\beta6$, $\alpha\beta3$, $\alpha\beta8$ and $\alpha\beta1$ have been reported as receptors for initiating wild-type FMDV infection in cell culture (Berinstein *et al.*, 1995, Jackson *et al.*, 2000, Jackson *et al.*, 2002, Jackson *et al.*, 2004). In cattle, $\alpha\beta6$ has been demonstrated as the major cellular receptor that determines viral tissue tropism *in vivo* (Monaghan *et al.*, 2005). Propagation of FMDV in cell culture results in the selection of variants with high affinity for heparin sulphate proteoglycans, a ubiquitous protein located at the external surface of cells (Jackson *et al.*, 1996). These tissue culture adapted viruses were previously thought to be less virulent in cattle compared to integrin binding isolates, however, this was shown not to be the case during the UK 2007 outbreak (Cottam *et al.*, 2008). Following multiple cell passages, viruses which do not bind heparin sulphate proteoglycans and lack the RGD integrin-binding motif still replicate efficiently in baby hamster kidney (BHK)-21 cells, suggesting that FMDV can adapt to an alternative unidentified surface receptor (Baranowski *et al.*, 2000). Following receptor binding, virus is taken up through clathrin-dependent endocytosis into the early and recycling endosomes (Berryman *et al.*, 2005). After uptake, the acidic environment in the endosome triggers the capsid to dissociate, the viral RNA is released and moves across the endosomal membrane into the cytoplasm by an unknown mechanism (Belsham, 2005, Berryman *et al.*, 2005).

1.2.3. Prevention and control of FMD

The control policies adopted by a particular country or region vary according to the FMD-status. The introduction of FMDV into a country previously classified as FMD-free usually results in attempts to eradicate the disease by slaughter so that the country can re-establish its FMD-free status for trade purposes. This was the policy adopted during the 2001 outbreak in the United Kingdom, although effective, the policy resulted in a massive overkill of healthy animals primarily due to delays in implementing movement restrictions. Public perception was that vaccination should be used in future outbreaks, however, during the 2007 outbreak in the United Kingdom, rapid and extensive movement restrictions and rapid diagnosis and slaughter effectively controlled the disease. If vaccinates are not slaughtered, a 12 month period was required before a country could re-apply for FMD-free status, the OIE reduced this period to 6 months in 2002, however, culling of infected and susceptible in-contact animals is still thought to be economically more viable in many situations. Control of the disease is further complicated when wildlife are involved and control policies in countries where the disease is endemic require a balance to support livestock-based initiatives and preserve the wildlife heritage in their natural ecosystems (Thomson *et al.*, 2003).

The current commercially available FMD vaccines commonly contain chemically inactivated FMDV as the antigen. The virus may be inactivated by, for example, treatment with aziridines which disrupt the RNA (Burrage *et al.*, 2000). Once inactivated the seed virus is blended with suitable adjuvant and excipients. Two categories of chemically inactivated vaccines are available, water based vaccines

adjuvanted with aluminium hydroxide and saponin, which are used for cattle, sheep and goats, and oil based vaccines which can also be used in pigs (Doel, 1999). The commercial vaccines are highly immunogenic and perform very well for regular vaccination programs and for control of outbreaks, however, the vaccines do not induce sterile immunity and protection is relatively short lived requiring a booster every 6 months to maintain immunity (Doel, 2003). Other limitations include thermal instability, lack of cross-protection between serotypes, risk of virus escape from production plants, absence of a defined chemical content which has been linked to anaphylactic shock and the difficulties distinguishing between infected and vaccinated animals (Barteling and Vreeswijk, 1991, Sobrino *et al.*, 2001). Therefore different approaches are being adopted to develop a safer and more effective vaccine.

1.3. The immune system and response to FMDV

The immune system can be broadly divided into the innate and adaptive immune systems. Interaction between the innate immune system, which responds quickly and non-specifically to a pathogen with recognition reliant on a limited number of germline-encoded receptors, and the adaptive immune response, which acts in an antigen-specific manner, is essential for the induction of an effective immune response to pathogens like FMDV (Palm and Medzhitov, 2009).

1.3.1. The innate immune system

During the early stages of infection, FMDV interacts with the innate immune system, a component of the host response to FMDV which has not yet received a significant amount of research. Consequently, in contrast to adaptive immunity, very little is

known about the contribution of innate immune defence during FMD. An effective, non-specific and rapid innate immune response is essential for the control of rapidly replicating, highly cytopathic and antigenically diverse viruses (Bachmann and Zinkernagel, 1997).

1.3.1.1. The complement system

As a first line of defence against pathogens, the complement system forms an important part of the innate immune response, able to activate cells involved both in the innate and adaptive immune response (Ricklin and Lambris, 2007). The complement cascade can be activated by three distinct pathways (Walport, 2001). The first pathway involves binding of C1q to antibody complexes on the surface of pathogens, activating the classical pathway. The related lectin pathway is activated when mannose-binding lectin interacts with mannose-containing carbohydrates on bacteria or viruses (Gadjeva *et al.*, 2001). The alternative pathway is initiated when the spontaneously activated complement component C3 binds directly to the surface of a pathogen (Favoreel *et al.*, 2003). Each pathway generates C3 convertase which results in the formation of the highly reactive C3b component which binds to the pathogens surface. This process, called opsonisation is critical for all subsequent steps in the complement cascade for elimination of pathogens (Favoreel *et al.*, 2003). Given the importance of complement as a central component of innate immunity, it is not surprising that mice deficient of important complement components like C3 are inefficient at controlling certain viral infections, for example influenza virus (Kopf *et al.*, 2002). Complement in early immune complexes can bind to

complement receptors and contribute substantially to antigen recruitment and facilitate B-cell activation (van Noesel *et al.*, 1993).

1.3.1.2. Type 1 interferons

The type 1 family of interferons (IFNs) are cytokines produced at the early stages of an immune response which are able to exert a vast array of biological functions including development and regulation of the innate and adaptive immune systems (Theofilopoulos *et al.*, 2005). Although virtually all cells can produce type 1 IFNs in response to pathogens and endogenous stimuli, plasmacytoid dendritic cells are the most potent and are referred to as “natural IFN-producing cells” (Colonna *et al.*, 2002). Current knowledge of the interactions of FMDV with plasmacytoid dendritic cells is discussed under section 1.3.1.5.

It has been demonstrated that mRNA encoding for type 1 IFN is induced within FMDV-infected cells *in vitro* and *in vivo*, however it is unclear if this message is translated into protein (Brown *et al.*, 2000, Chinsangaram *et al.*, 1999, Zhang *et al.*, 2009, Zhang *et al.*, 2006). FMDV can shut down protein synthesis through the activity of the viral L^{pro} which cleaves the translation initiation factor eIF4G, a factor essential for CAP-dependent mRNA translation (Devaney *et al.*, 1988, Medina *et al.*, 1993). The viral L^{pro} is a feature unique to the aphthovirus genus of the *Picornaviridae* family (Hinton *et al.*, 2002) and L^{pro} interference with host protein synthesis has been proposed as an important evolutionary immune evasion technique, counteracting the innate immune response (de Los Santos *et al.*, 2008). L^{pro} plays a critical role in FMD pathogenesis and viruses lacking this coding region are

attenuated *in vitro* and *in vivo* (Brown *et al.*, 1996). Blocking host translation is particularly relevant for IFN expression since FMDV is highly sensitive to the actions of type 1 IFNs *in vitro*, with IFN induced dsRNA protein kinase and ribonuclease L shown to inhibit replication (Chinsangaram *et al.*, 2001, de Los Santos *et al.*, 2006). In addition, type 1 IFNs can protect pigs against challenge infection highlighting the importance of IFN during the innate immune response to FMDV (Chinsangaram *et al.*, 2001, Chinsangaram *et al.*, 2003, Grubman, 2005). Studies in our laboratory have recently identified significant titres of biologically active type 1 IFN in the circulation of FMDV contact-infected cattle (unpublished data) demonstrating that translation of type 1 IFN is not completely blocked in all cell types that are infected *in vivo* (see section 1.3.1.5).

1.3.1.3. Natural antibodies

Natural antibodies are low-affinity, polyreactive antibodies in the sera of normal, non-immunised individuals, detected even under germ-free conditions (Haury *et al.*, 1997, Ochsenbein and Zinkernagel, 2000). The B1 B-cells in mice produce natural antibodies (see section 1.3.2.1). Natural antibodies are considered as a link between the innate and adaptive immune responses, able to limit pathogen dissemination and forming immune complexes to activate adaptive immunity, recruit antigen to follicular dendritic cells in organised lymphoid tissue (see section 1.4) and activate complement (Dörner and Radbruch, 2007). Virus neutralising titres of natural antibodies have been identified, for example, natural antibodies have been detected in mice that can directly neutralise the highly cytopathic vesicular stomatitis virus (VSV), (Hangartner *et al.*, 2006). The importance of natural antibodies for an

effective immune response, specifically for responses against cytopathic viruses, is highlighted by impaired immune protection in mice lacking natural antibodies and challenged with influenza virus (Baumgarth *et al.*, 2000). There are no reports of natural antibodies in cattle or of natural antibodies directed against FMDV. However, anecdotal evidence of nonspecific background in non-immunised cattle detected by immunological assays in the FMDV World Reference Laboratory, Pirbright, supports a case for further investigation.

1.3.1.4. Macrophages and neutrophils

Macrophages and neutrophils are important, not only for phagocytosis and killing of pathogens but also for antigen presentation, therefore forming an important connection between the innate and adaptive immune systems (Sandilands *et al.*, 2005). Recognition and uptake of pathogens by macrophages is restricted by a number of phagocytic receptors including fragment crystallisable (Fc) receptors and complement receptors. Ligand interaction with these receptors also induces the production of cytokines and chemokines that stimulate other cells, for example, dendritic cells to migrate to the site of infection (Aderem and Underhill, 1999). Phagocytosis induced by Fc receptors results in the production and secretion of reactive oxygen intermediates and arachidonic acid metabolites, in contrast, complement receptor mediated phagocytosis does not (Aderem *et al.*, 1985, Wright and Silverstein, 1983). It has been reported that porcine macrophages take up FMDV *in vitro*, a process enhanced in the presence of antibody-virus complexes (McCullough *et al.*, 1988, Rigden *et al.*, 2002). During the first 10 hours post-infection these cells contain non-structural viral proteins and release small quantities

of virus, although it is not clear if this represents progeny virus released before the productive virus replication cycle is aborted or exocytosed uptake virus (Rigden *et al.*, 2002).

1.3.1.5. Dendritic cells

Dendritic cells (DCs) can be broadly divided into 2 major subsets, conventional DCs and plasmacytoid dendritic cells (pDCs). DCs are an important member of the antigen presenting cell family, unique in their ability to stimulate naïve T cells (Kapsenberg, 2003). Like macrophages they are highly endocytic and constantly sample their environment through both receptor-mediated and non-specific routes of endocytosis. DCs are distributed in the body in both lymphoid and non-lymphoid tissues forming a vast sentinel system able to respond to foreign antigen by expressing pattern recognition receptors both on the surface and within endocytic compartments (Lee and Kim, 2007). DCs are also able to react to conditions of injury or infection by responding to a number of inflammatory mediators including pro-inflammatory cytokines, called “danger signals” which promote DC maturation and migration (Gallucci and Matzinger, 2001). The process of maturation is essential for effective antigen presentation to lymphocytes in lymphoid tissue (Banchereau and Steinman, 1998). As DCs mature they efficiently capture antigen and express major histocompatibility complex (MHC) class I and II-peptide complexes and high-levels of co-stimulatory molecules on their surface (van Vliet *et al.*, 2007). Maturation also results in migration to the lymph tissue and a change of morphology to the characteristic form with highly dendritic processes, increasing the cell surface area and allowing intimate contact with T cells (Banchereau and Steinman, 1998). DCs

can also take up and maintain intracellular pools of undegraded antigen (Wykes *et al.*, 1998). The undegraded antigen can be transported to draining lymph nodes and recycled to the cell surface for engagement with B cells that recognise the intact protein (Qi *et al.*, 2006). The location of DCs within lymph nodes of mice varies according to their origin. Resident DCs are sessile, they are localised throughout the lymph node but are concentrated in the cortical ridge where they actively probe passing motile T cells (Cavanagh and Weninger, 2008). Freshly migrated DCs carry antigen from the periphery and traverse through the cortex of the draining lymph nodes scanning for T cells (Mempel *et al.*, 2004). Migratory Langerhans-derived DCs populate the deeper cortex, whereas dermal DCs localise to the cortical ridge at the T-B cell border where they continually scan T cells or near the high endothelial venules (HEV) where they encounter newly homed T cells (Cahalan and Parker, 2008, Cavanagh and Weninger, 2008).

DCs have a dual role, they are capable of inducing an effector immune response or they can maintain tolerance by either inducing cells with immune-suppressive functions or by deleting and suppressing certain T-cell clones (Steinman and Banchereau, 2007). DCs therefore comprise a diverse and complex subset of cells that differ from one another in terms of location, antigen presentation, state of maturation and interaction with different lymphocyte populations, making them an extremely difficult population of cells to study (Banchereau and Steinman, 1998). Consequently, there are conflicting results of DC interaction with FMDV in the literature, in addition all the studies reported so far have been performed with either murine or porcine derived cells and not bovine cells (Summerfield *et al.*, 2008). It is

clear from these studies that DCs do take up FMDV, a process which can be enhanced in the presence of FMDV-specific antibody. Uptake is also enhanced for cell culture adapted viruses, which can bind and infect cells via surface expressed heparin sulphate structures (Jackson *et al.*, 1996). However, it is not clear how susceptible the different subsets of DCs are to infection and what affect this has on the way DCs interact with other cells (Bautista *et al.*, 2005, Gregg *et al.*, 1995). Following FMDV infection of DCs, non-structural viral proteins and double-stranded RNA can be detected for up to 24 hours post-infection. In addition, small quantities of virus are released between 2 and 8 hours post-infection. However, as is the case for macrophages, it is not clear if this represents progeny virus released before the productive virus replication cycle is aborted or exocytosed uptake virus (Harwood *et al.*, 2008). Studies in our laboratory (Robinson *et al.* manuscript in preparation) have recently described the interactions of FMDV with bovine cells generated from cluster of differentiation (CD) 14⁺ PBMC. FMDV was able to infect bovine monocyte-derived macrophages and DCs and infection was enhanced in the presence of specific antibody and cell culture adapted virus, similar to the results reported above for DCs isolated from other species. However, it is still unclear if the infection is productive and further studies are required for clarity. FMDV infection of bovine monocyte-derived DCs results in cell death and as a consequence, the amount of antigen processed and presented by DCs to T cells is reduced, as determined by proliferation assays, highlighting the importance of understanding the interaction of FMDV with DCs (Robinson, 2008).

pDCs were first described in humans as a subset of cells specialised in the secretion of type 1 IFNs in response to certain viruses (Fitzgerald-Bocarsly, 1993, Lennert and Remmele, 1958). These lymphoid derived cells were identified on the basis of their plasma-cell-like morphology and expression of CD4, their ability to stimulate helper T cells and their location in the T-cell areas of lymph nodes (Colonna *et al.*, 2002). pDC have also been described in human skin (Zaba *et al.*, 2007) and in the lung, liver and blood of mice (Abe *et al.*, 2004, de Heer *et al.*, 2004, Diacovo *et al.*, 2005). pDCs respond to microbial nucleic acids during infection, in addition, when there is a breakdown of innate tolerance they can respond to self nucleic acid which can trigger autoimmune diseases, for example, systemic lupus erythematosus (Gilliet *et al.*, 2008). In order to discriminate between pathogen derived and self nucleic acids, pDCs do not express receptors for nucleic acids on their surface but rely on the subcellular localisation of Toll-like receptors (TLR) to respond to pathogens that invade by endocytosis. Endosomal TLR7 is required to respond to single-stranded RNA viruses like influenza virus or VSV (Lund *et al.*, 2004) and endosomal TLR9 expression is required to respond to single-stranded DNA molecules. In addition, TLR9 is only activated by single-stranded DNA molecules that contain unmethylated CpG-containing motifs, which are commonly found in the genomes of DNA viruses such as herpesviruses and in bacteria (Gilliet *et al.*, 2008, Krug *et al.*, 2004). Unlike DCs, pDCs do not express TLR2, TLR4, TLR5 or TLR3, which explains why they do not respond to bacterial products such as peptidoglycans, lipopolysaccharide and flagellin, or viral double-stranded RNA (Colonna *et al.*, 2004).

pDC homologs have been described in pigs (Domeika *et al.*, 2004, Summerfield *et al.*, 2003, Riffault *et al.*, 2001) and in sheep (Pascale *et al.*, 2008). They have been identified in the skin and at mucosal surfaces of these two species where they are able to interact with invading pathogens, for example, transmissible gastroenteritis virus infection in piglets (Riffault *et al.*, 2001). In addition, pDCs are able to migrate in afferent lymph to the draining lymph node, enabling presentation of antigen captured at peripheral sites (Pascale *et al.*, 2008). Interactions with FMDV have been investigated with porcine blood derived pDCs (Guzylack-Piriou *et al.*, 2006). FMDV was shown to undergo a similar abortive replication cycle in porcine pDCs as it does in DCs. However, infection was only initiated in the presence of specific antibody and associated with CD32 expression (Guzylack-Piriou *et al.*, 2006). Type 1 IFN induction was dependent on FMDV replication and the authors concluded that the response was mediated by receptors associated with the endocytic process, for example, TLR7 (Guzylack-Piriou *et al.*, 2006). Cells have been identified in bovine lymph nodes that are capable of producing type 1 IFN in response to noncytopathic bovine viral diarrhoea virus (Brackenbury *et al.*, 2005). However, these cells expressed myeloid markers and did not express CD4 or CD45RB suggesting that they were not the bovine homolog of pDCs. Researchers have not yet identified pDCs in cattle, other cell types, including monocytes and B cells are also capable of producing type 1 IFN in response to viral infections (Fitzgerald-Bocarsly, 2002) and the cellular source of the biologically active type 1 IFN detected in FMDV infected cattle is yet to be determined.

1.3.1.6. Natural killer cells

Natural killer (NK) cells are bone marrow derived lymphoid cells that are capable of lysing tumour cells and virus-infected cells without prior sensitisation (Yokoyama *et al.*, 2004). NK cells are activated either by cytokine stimulation, for example, by Interleukin (IL) -12 produced by activated macrophages and DCs (Gerosa *et al.*, 2002, Yokoyama *et al.*, 2004) or by target cell recognition. NK cells are able to discriminate between healthy cells and target cells, recognising and killing infected cells or tumour cells by a complicated process mediated by the concomitant action of activating and inhibitory receptors (Lanier, 2005). Some of the inhibitory receptors recognise MHC class I, which is present on most healthy cells thereby dampening NK-cell activity and preventing attack (Lanier, 2005). Activated NK cells lyse virus infected or tumour cells in the same way as CD8⁺ cytotoxic T cells, a process mediated by perforin pores and granzyme (Biron and Brossay, 2001). In addition, NK cells produce a number of cytokines, for example TNF- α and IFN- γ , both of which are important modulators of the immune response, capable of inducing DCs and macrophages (Walzer *et al.*, 2005).

The interaction of FMDV with NK cells is a neglected field of research, primarily because NK cells have only recently been identified and characterised in ruminant (Storset *et al.*, 2004). The only evidence to support a putative role for NK cells in FMDV pathogenesis in bovines stems from studies of cells with an NK-cell like phenotype, derived from FMDV restimulated PBMC of vaccinated cattle (Amadori *et al.*, 1992). These IL-2 stimulated CD45⁺ cells were able to lyse FMDV-infected target cells in a non-MHC restricted manner (Amadori *et al.*, 1992). It has also been

suggested that NK cell activity could play an important role in FMD during viral down-regulation of MHC class I on infected epithelium (Sanz-Parra *et al.*, 1998). However, it has also been suggested that down-regulating MHC class I may be part of the viral immune evasion strategy to prevent cytolysis by MHC class I-restricted T lymphocytes (Grubman *et al.*, 2008, Summerfield *et al.*, 2008). Recently, it was shown that a population of non-adherent porcine PBMC enriched for NK cells by negative selection, were able to lyse FMDV infected porcine kidney fibroblasts *in vitro* after stimulation with proinflammatory cytokines (Toka *et al.*, 2009). The fibroblasts were infected with an attenuated, heparin sulphate binding strain of FMD, LL-KGE which lacks the L^{pro}. The greatest lytic capacity was seen after incubation with IL-2 or IL-15. Lower activation was induced by IL-12, IL-18 or IFN- α , however combining IL-12 and IL-18 increased the lytic capacity of these cells. These data suggest that the porcine innate immune response against FMDV can be enhanced by proinflammatory cytokines (Toka *et al.*, 2009). The recent characterisation of an antibody directed against bovine NK cells, NKp46, should lead to more detailed studies of NK cell function and the role of these cells in FMD pathogenesis (Storset *et al.*, 2004).

1.3.1.7. Gamma delta T cells

The $\gamma\delta$ T cells account for a relatively large proportion of the lymphocyte population in ruminants, with even greater numbers (50% of the lymphocytes in circulation) reported in juvenile animals (Clevers *et al.*, 1990, Pollock and Welsh, 2002). Like $\alpha\beta$ T cells the $\gamma\delta$ T cells express a T-cell receptor (TCR) on their surface which recognises antigen. The bovine TCR, as is the case for other animals, is associated

with up to 5 non-covalently linked invariant components termed the CD3 γ , δ , and ϵ and TCR ζ and η chains and together they form the TCR complex (Pescovitz *et al.*, 1998). However, the majority of $\gamma\delta$ T cells lack the co-receptor molecules CD4 and CD8, which play an important role in MHC restricted activation of $\alpha\beta$ T cells (Cron *et al.*, 1989). Similar to the $\alpha\beta$ TCR, each chain of the heterodimeric $\gamma\delta$ TCR comprises of an immunoglobulin like extracellular domain with a variable and constant region, a transmembrane segment and a cytoplasmic domain. However, sequence analysis in humans has revealed that the $\gamma\delta$ TCR is more closely related to surface expressed immunoglobulin's on B cells and structural analysis has revealed fundamental differences in the extracellular domain when compared to the $\alpha\beta$ TCR (Allison *et al.*, 2001). The main differences exist in the third complementary-determining region (CDR3) loop of the TCR, a region which interacts directly with antigenic peptides (Nishio *et al.*, 2004). This region of the $\gamma\delta$ TCR has been shown to be longer and more variable than the $\alpha\beta$ TCR in humans and in mice (Rock *et al.*, 1994). These differences allow antigens and damaged tissue to interact directly with the $\gamma\delta$ TCR without the requirement for MHC molecules and protein processing pathways (Schild *et al.*, 1994, Rock *et al.*, 1994). However, this is not the case for all $\gamma\delta$ T cells because the small percentages of $\gamma\delta$ T cells which express CD4 or CD8 in humans and mice depend on antigen processing pathways and presentation by MHC molecules by cause of the restrictions in antigen interaction by CDR3 shortening in both CD4⁺ and CD8⁺ thymocytes (Haas *et al.*, 1993, Nishio *et al.*, 2004). Although a defined role for the $\gamma\delta$ T cells remains unclear, these cells have been attributed as a first line of defence with other innate immune responses and seem to be biased towards the recognition of certain types of microbial antigens (Hayday, 2000). It is

unclear if these cells are able to display immunological memory and participate in recall responses (Blumerman *et al.*, 2007). There is evidence in humans and mice that $\gamma\delta$ T cells can undergo antigen priming, altering the cellular responsiveness on secondary encounter with the antigen (Hoft *et al.*, 1998, Spaner *et al.*, 1993).

Similarly in cattle, *in vivo* priming with killed *Leptospira* vaccine has been shown to alter the cellular response of a subset of $\gamma\delta$ T cells on re-encounter with the antigen *in vitro*. Priming was associated with a larger percentage of $\gamma\delta$ T cells undergoing blastogenesis *in vitro* compared to cells from naïve animals, suggestive of a memory-like phenotype (Blumerman *et al.*, 2007).

Two distinct populations of $\gamma\delta$ T cells have been characterised in cattle based on their cell-surface phenotype and tissue distribution. Workshop cluster (WC) 1 is a transmembrane glycoprotein, uniquely expressed on $CD2^-/CD4^-/CD8^-$ $\gamma\delta$ T cells (Carr *et al.*, 1994, Clevers *et al.*, 1990). In cattle, $WC1^+$ $\gamma\delta$ T cells represent less than 10% of the mononuclear cell population in the lymph node, thymus and spleen and represent between 10 to 15% of the PBMC, with higher percentages reported in juvenile animals (MacHugh *et al.*, 1997). The $WC1^-$ subset expresses CD2 and CD8. The majority of bovine $WC1^-$ $\gamma\delta$ T cells reside in the red pulp of the spleen where they are reported to represent approximately 30% on the mononuclear cell population (MacHugh *et al.*, 1997).

Three isoforms of WC1, a protein associated with $\gamma\delta$ T cells growth arrest, have been identified in ruminants, WC1.1, WC1.2 and WC1.3 (Hanby-Flarida *et al.*, 1996, Pillai *et al.*, 2007, Takamatsu *et al.*, 1997). Bovine $WC1.1^+$ and $WC1.2^+$

subpopulations have been shown to act as regulatory cells *ex vivo* and express IL-10, potentially playing an important role for maintenance of both innate and antigen specific adaptive immune responses (Ferrick *et al.*, 1995, Hoek *et al.*, 2009). WC1⁺ $\gamma\delta$ T cells have been found to play a role in the immune response against bacterial, parasitic and viral infections in cattle. The majority of evidence for the role of WC1⁺ $\gamma\delta$ T cells in cattle is based on studies of the immune response to bacterial infections, for example *Mycobacterium bovis*, *Leptospira* species and staphylococci (Fikri *et al.*, 2001, Kennedy *et al.*, 2002, Naiman *et al.*, 2002) where they have been shown to proliferate and produce the cytokines IL-12, IFN- γ and TNF- α . Proliferation and transcription of cytokines has also been reported in response to parasitic infections including *Theileria annulata*, *Theileria parva*, in addition, NK-like cytotoxicity has been reported following *in vitro* exposure to *Babesia bovis* (Brown *et al.*, 1994, Collins *et al.*, 1996, Daubenberger *et al.*, 1999). The response of WC1⁺ $\gamma\delta$ T cells in ruminants to viral infections has not been extensively investigated and little is known about the involvement of these cells in viral pathogenesis. There are reports of a regulatory role during immune responses to viral infections with enhanced antibody responses detected following respiratory syncytial virus challenge in WC1⁺ depleted calves (Taylor *et al.*, 1995). These cells have also been shown to increase in circulation following challenge with bovine leukaemia virus, however the significance of this response is unclear (Ungar-Waron *et al.*, 1996). Purified, naïve porcine WC1⁺ $\gamma\delta$ T cells are able to respond directly to FMDV antigen, a response characterised by proliferation and increased expression of pro-inflammatory cytokines and chemokines (Takamatsu *et al.*, 2006). There are no reports in the

literature on the response of bovine $\gamma\delta$ T cells to FMDV and a role for these cells in FMD pathogenesis has not been investigated.

1.3.2. The adaptive immune system

1.3.2.1. Humoral immunity

Humoral immunity is the component of the adaptive immune response mediated by antibody produced by B cells. B cells are generated in the bone marrow and recognise antigen through the antigen specific B-cell receptor which is formed by somatic recombination of germline encoded genes (Murre, 2007). Bovine B cells can be divided into two subsets, B1 and B2 B-cells. Bovine B1 B-cells are considered a more primitive cell type and express the antigens CD5, a molecule implicated in the negative regulation of B-cell-receptor signalling (Lenz, 2009) and CD11b, a receptor for the proteolytically inactive product of the complement cleavage fragment C3b (Michishita *et al.*, 1993). The majority of these cells are L-selectin⁻ and subsequently do not recirculate through the lymph nodes and can be found predominantly in the pleural and peritoneal cavities (Howard and Morrison, 1994, Naessens and Williams, 1992). In contrast, the L-selectin⁺ B2 B-cells, considered to be conventional B cells, recirculate through lymph nodes and do not express CD5 or CD11b (Howard and Morrison, 1994, Naessens and Williams, 1992). The B1 B-cells in mice are responsible for producing natural antibodies (see section 1.3.1.3) and together with mouse splenic marginal zone B cells are classified as “innate B lymphocytes”, acting as a first line of defence against invading pathogens (Carey *et al.*, 2008, Kearney, 2005). These cells express mostly immunoglobulin (Ig) M and are involved in T-independent (T-I) antibody responses (Howard and Morrison, 1994, Ostrowski *et al.*,

2007). Antigens that are able to stimulate naïve B cells in the absence of T cell help are known as T-I antigens (Obukhanych and Nussenzweig, 2006). The T-I antigens can be further subdivided into type I and type II T-I antigens. Type I T-I antigens are mitogenic agents, for example, lipopolysaccharides, unmethylated CpG and polyriboinosinic: polyribocytidylic acid (poly IC), that activate TLRs to elicit polyclonal B cell activation. Type I T-I antigens are generally considered to be more potent B cell stimulators than type II T-I antigens and are able to activate immature B cells (Cambier *et al.*, 1994, Obukhanych and Nussenzweig, 2006, Scher, 1982). Type II T-I antigens are typically complex, rigid structures that engage and cross-link the immunoglobulin receptors on the surface of B cells generating strong activation signals to produce antibody, in the absence of specific T cell help (Obukhanych and Nussenzweig, 2006). The repetitiveness and degree of antigen organisation determines whether the antigen can generate a strong enough signal to induce antibody production or if there is a requirement for accessory signals from antigen presenting cells or T cells (Cambier *et al.*, 1994). Interaction of the B-cell surface immunoglobulin receptor with T-dependent (T-D) antigens leads to activation of a cascade of protein kinases and antigen internalisation (Cambier *et al.*, 1994). The antigen is processed and presented on MHC class II molecules, however, antibody is not produced and the B cell does not undergo proliferation. This mechanism of uptake by a B cell is highly efficient and B cells constitutively express high levels of MHC class II molecules. A successful B-cell response to a T-D antigen is dependent on encounter with a primed CD4⁺ T cell since B cells will tolerise naïve T cells (Cambier *et al.*, 1994, Eynon and Parker, 1992). The costimulatory molecules B7.1 (CD80) and B7.2 (CD86) are upregulated on encounter with a specific, primed

helper T cell (June *et al.*, 1994). These molecules interact with CD28 on T cells, leading to CD40 ligand (CD154) expression. The CD40-CD154 interaction induces B-cell proliferation, antibody production and isotype class switching (Armitage *et al.*, 1992). The B cell co-receptor complex CD19:CD21:CD81 is also an important component of B-cell activation, coupling the innate complement system with B-cell activation (Fearon and Carroll, 2000). CD21 is a receptor for the complement fragment C3d, an interaction which increases B-cell responsiveness (Carter *et al.*, 1988). However, it is not clear if the increased responsiveness is a result of increased B-cell signalling, the induction of co-stimulatory molecules on the B cell or increased receptor mediated uptake of antigen (Fearon and Carroll, 2000).

A number of cytopathic viruses, for example VSV (Battegay *et al.*, 1996), influenza virus (Lee *et al.*, 2005) and rotavirus (Franco and Greenberg, 1997) have been described to act as T-I antigens in mice. The rapid induction of a protective immune response directed against these acute cytopathic viruses is essential to ensure host survival by controlling virus spread through systemic circulation (Bachmann and Zinkernagel, 1997). The capacity of these viruses to induce a T-I antibody response, characterised by a rapid and potent IgM response, is associated with the high organisation of viral surface antigens (Bachmann and Zinkernagel, 1996). FMDV is able to induce a rapid and specific T-I neutralising antibody response in mice (Borca *et al.*, 1986, Lopez *et al.*, 1990), a response mediated, at least in part, by splenic innate B cells (Ostrowski *et al.*, 2007). However, it is unknown whether this response exists in any natural host of FMDV. The importance of humoral immunity in FMD is well documented, over a hundred years ago it was demonstrated that antibodies form

the major mechanism of protection against FMDV using passive transfer experiments in cattle (Loeffler and Frosch, 1897). Because of the importance of antibody, a number of studies have examined the classes and subclasses of virus neutralising antibody in serum and probang samples of cattle. Specific IgM is detected in the serum between 3 to 7 days after challenge, reaching a peak between 5 and 14 days then slowly declining to an undetectable level at the latest 56 days post-infection. Isotype switching occurs rapidly with specific IgG1 and IgG2 detected from 4 days post-challenge and reach maximal levels between 14 and 20 days (Collen, 1994, Doel, 2005, Salt *et al.*, 1996a). Virus neutralising antibody has been detected up to 4.5 years after experimental infection in bovines (Cunliffe, 1964). IgA is initially detected in serum and probang samples from 7 days after challenge with a peak titre detected at 7 to 14 days in serum and an initial peak titre at 14 days in probang samples. The IgA titre in serum slowly declines from 14 days except in ‘carriers’ where a significant second late response beginning at 28 days is detected. A second late response is detected from day 28 in probang samples of all infected cattle independent of their ‘carrier’ state. The IgA titre in probang samples either decline to undetectable levels or persist in animals classified as ‘carriers’ (Salt *et al.*, 1996a). The titre of secretory IgA has been considered as a tool for identifying ‘carrier’ animals and for detecting sub-clinical infection in vaccinated cattle (Parida *et al.*, 2006).

An effective immune response against FMDV is characterised by the induction of high titres of antibody. Although there is a close correlation between FMDV serum neutralising antibody titres (SNTs) and protection from infection, this correlation is

not precise (McCullough *et al.*, 1992). This imprecise correlation is highlighted in certain vaccine potency testing studies during which animals with low or no detectable neutralising antibody titre were resistant to challenge while others with acceptable titres were susceptible (Barnett and Carabin, 2002). This disparity could be explained by different neutralisation mechanisms *in vivo* in the presence of other immune system components compared to the *in vivo* FMDV neutralising antibody test used to determine the antibody titres. The ability of antibody to neutralise virus *in vivo* is far more complex, involving the interaction of antibody with cells and molecules of the innate immune system and under these conditions non-neutralising antibody can contribute to protection (Reading and Dimmock, 2007). The described mechanisms of FMDV neutralisation *in vitro*, as determined by the virus neutralising antibody test, includes inhibition of cell attachment leading to loss of infectivity due to steric hindrance with integrin interactions or destabilisation of the virus capsid, which leads to premature uncoating and particle destruction (McCullough *et al.*, 1992, McCullough *et al.*, 1987b). It is noteworthy that the 4C9 destabilising MAb described by McCullough *et al.* could disrupt the virion capsid at 37°C under normal ionic conditions, in contrast to MAbs described for poliovirus which could only irreversibly inactivate poliovirus at temperatures above 39°C or in a low-ionic-strength environment (Delaet and Boeye, 1993, McCullough *et al.*, 1987b).

A number of antibody-mediated mechanisms that inhibit virus attachment or virus cell entry events have been described. Antibodies can block the cell attachment site on the virus particle or induce aggregation (Brioen *et al.*, 1983). In addition, it has been hypothesised that a single antibody molecule can induce conformational

changes in crucial capsid molecules which can block virus attachment or block post-entry events, for example, preventing virus uncoating by cross-linking the capsid as demonstrated for a MAb directed against human adenovirus (Reading and Dimmock, 2007, Wohlfart *et al.*, 1985). However, the role of antibody in blocking late steps in entry is largely unknown. Recently, a new mechanism by which antibodies block virus infection has been described for human adenovirus (Smith *et al.*, 2008). Human adenovirus is a nonenveloped DNA virus that interacts with cellular integrins through a conserved RGD motif in addition to the adenovirus receptor CD46 and is taken up through clathrin-dependent endocytosis (Wickham *et al.*, 1993). A neutralising antibody has been described that blocks infection *in vitro* by inhibiting virus microtubule-dependent translocation from the site of endosome penetration through the cytoplasm to the nuclear envelope (Smith *et al.*, 2008).

An antibody occupancy model to block virus entry has also been proposed (Burnet *et al.*, 1937). According to this model, virus attachment or entry into the host cell is inhibited when a large proportion of the epitopes on the virion are occupied by antibody which increases the size of the virus particle (Burton, 2002). This model highlights the importance of high affinity antibody directed against epitopes on the virion surface at sites not involved with cell-receptor recognition (Burton, 2002). Binding of a single IgM molecule or two closely spaced IgG antibodies to a virus can also activate the classical pathway of the complement system by binding of C1q to the immune complex (Spear *et al.*, 2001). As complement activation proceeds at the virus surface, there is a build-up of complement components which coat the virus, interfering with virus binding, as shown *in vitro* with avian infectious bronchitis

virus (Berry and Almeida, 1968). In addition, as the membrane attack complex of complement is activated, pores are formed in the membrane of enveloped viruses, for example human immunodeficiency virus (HIV) type-1, leading to virolysis (Sullivan *et al.*, 1996). Fc and complement receptors can also bind the immune complexed virus which leads to phagocytosis and virus inactivation. This process has been described *in vitro* for FMDV and the protective immune response against FMDV *in vivo* is thought to be dependent on the interaction between antibody-virus complexes and the phagocytic cells of the reticuloendothelial system (McCullough *et al.*, 1986, McCullough *et al.*, 1992, McCullough *et al.*, 1988). Antibody-complexing of virus can also enhance infection of Fc receptor bearing cells, for example, enhancement of Dengue virus infection *in vitro* is mediated by Fc receptors (Boonnak *et al.*, 2008, Halstead, 1982), a process that may also enhance infection *in vivo* (Goncalvez *et al.*, 2007).

Antibody can also interact with infected cells by binding viral proteins that are expressed on the cell surface. Binding of antibody to infected cells can lead to cell lysis or clearance by Fc-mediated antibody-dependent cellular cytotoxicity or complement dependent cytotoxicity (Burton, 2002). Binding of antibody to viral molecules on the cell surface has also been shown to inhibit viral replication within the cell, for example, clearance of alphavirus infection from rat neurons *in vitro* (Levine *et al.*, 1991). In addition, virus release from the infected cell and cell-to-cell transmission can be inhibited, for example, antibodies directed against influenza virus transmembrane protein can reduce virus yield (Gerhard, 2001, Reading and Dimmock, 2007). Generally, antibody functions against extracellular and cell surface

antigen whereas cell-mediated immunity forms a surveillance system for intracellular pathogens. However, polymeric IgA and IgM are the exception and can mediate intracellular neutralisation of viruses, for example, HIV transcytosis can be blocked *in vitro* by IgA and IgM specific for envelope proteins leading to intracellular virus neutralisation (Bomsel *et al.*, 1998). In addition, non-neutralising IgA can protect against rotavirus infection in mice *in vivo* by a similar mechanism (Burns *et al.*, 1996).

1.3.2.2. Cell mediated immunity

Cell mediated immunity describes the effector function of T lymphocytes that serve as a defence against intracellular pathogens. Classical antigen recognition by $\alpha\beta$ T cells is mediated by the $\alpha\beta$ TCR complex which recognises processed antigenic-peptide presented on the surface of antigen presenting cells or infected cells by MHC molecules (Roitt and Delvis, 2001). The $\alpha\beta$ TCR, like the immunoglobulin receptor of B cells, undergoes somatic recombination of germline encoded genes resulting in numerous antigen specific TCRs. Antigen can be presented to T cells by four types of antigen-presenting cell, monocytes, macrophages, DCs which are able to present antigen and stimulate naïve T cells and B cells which present antigen fragments recognised by their surface immunoglobulin (Trombetta and Mellman, 2005).

Classically, it is considered that proteins synthesised intracellularly such as viral proteins are degraded and presented by MHC class I molecules to cytotoxic $CD8^+$ T cells whereas extracellular proteins are presented by MHC class II molecules to $CD4^+$ T cells (Germain, 1994), however, it is now recognised that additional, alternative routes exist for proteins to be presented, including cross-presentation and

autophagy (Cresswell, 2005). Once the TCR is engaged with an antigen of the correct specificity it receives the first TCR complex activation signal. The T cell will only be activated if it receives the second activation signal involving the interaction of CD28 on the T cell and B7.1 and B7.2 on the antigen presenting cell. If the T cell does not receive this second signal it becomes anergic. Activation results in the production of IL-2 which induces clonal expansion in an autocrine manner (Colombetti *et al.*, 2006). The T cells then differentiate into effector cells and memory cells (see section 1.6.1).

The CD4 molecule consists of a single polypeptide belonging to the immunoglobulin gene superfamily, with CD4⁺ T cells representing approximately 24 to 35% of PBMC in cattle (Howard and Morrison, 1994). As for other species, CD4⁺ T cells in ruminants are MHC class II restricted (Baldwin *et al.*, 1986). Depletion experiments in cattle, targeting CD4⁺ cells with specific mouse MAbs, have demonstrated that these cells are essential for producing antibody to T-D antigens (Howard *et al.*, 1989). The progeny of antigen stimulated CD4⁺ T cells differentiate into effector cells that can activate macrophages, cytotoxic CD8⁺ T cells and B cells.

A role for CD4⁺ T cells during the immune response against FMDV has not yet been defined. FMDV is able to induce a rapid and specific T-I neutralising antibody response in mice (Borca *et al.*, 1986, Lopez *et al.*, 1990). However, it is not clear if T cells are required to induce a protective neutralising antibody response in cattle. FMDV-specific CD4⁺ T-cell-proliferative responses are detectable following infection or vaccination with virus or peptide (Blanco *et al.*, 2001, Collen and Doel,

1990, Gerner *et al.*, 2007) and several haplotype-restricted and “promiscuous” CD4⁺ T cell epitopes have been identified on both the structural and non-structural proteins suggesting that cell-mediated immunity may be involved in the immune response (Blanco *et al.*, 2000, Collen and Doel, 1990, Gerner *et al.*, 2007, van Lierop *et al.*, 1995). Current work in our group (Windsor *et al.*, manuscript in preparation) has detected CD4⁺ T-cell-proliferative responses to vaccine antigen following primary FMDV O UKG infection in cattle. However, these responses are usually variable and of low magnitude. These reduced responses are not a consequence of generalised immunosuppression during infection because recall responses to unrelated antigens are unaffected, therefore bringing into question the contribution by CD4⁺ T cells to the immune response and memory response after primary FMDV infection.

The CD8 molecule, which also belongs to the immunoglobulin gene superfamily, is usually expressed as a noncovalently linked heterodimer consisting of α and β chains. However, homodimers of only the α chain can exist, which is the chain involved in binding to MHC class I molecules through its immunoglobulin like extracellular domain (Howard and Morrison, 1994). CD8⁺ T cells represent approximately 15 to 25% of PBMC in cattle (Howard and Morrison, 1994). The CD8⁺ T cells differentiate into effector cytotoxic T lymphocytes and mediate MHC class I restricted cytotoxicity against infected cells with help from CD4⁺ T cells. Depletion experiments in cattle have demonstrated the importance of these cells in viral infections like respiratory syncytial virus and rotavirus where they play a major role in resolution of the primary infection (Oldham *et al.*, 1993, Taylor *et al.*, 1995). The role of CD8⁺ T cells in FMDV infection is also unclear. Recently, FMDV-

specific MHC class I restricted CD8⁺ T cells were detected in cattle, following both infection and vaccination, using an IFN- γ restimulation ELISpot assay (Guzman *et al.*, 2008). As discussed under section 1.3.1.6, FMDV down regulates MHC class I on infected epithelial cells (Sanz-Parra *et al.*, 1998). MHC class I expression is reduced by approximately 50% just 6 hours post-infection, potentially effecting the ability of CD8⁺ T cells to recognise and eliminate infected cells (Grubman *et al.*, 2008).

1.4. Follicular dendritic cells

Follicular dendritic cells (FDCs) (Chen *et al.*, 1978) are specialised, non-endocytic, immune accessory cells found in the follicles of organised lymphoid tissue (Allen and Cyster, 2008, Sukumar *et al.*, 2008). Although morphologically heterogeneous, a factor attributed to differences in maturity (El Shikh *et al.*, 2006, Szakal *et al.*, 1989), FDCs characteristically possess long, delicate cytoplasmic extensions which form a reticular network in close contact with adjacent lymphocytes. They are also characterised by electron-lucent vesicles in the cytoplasm and deeply indented or bilobed euchromic nuclei (Sukumar *et al.*, 2008). A particular striking feature of FDCs is their ability to trap and retain antigen in the form of immune complexes on the surface of their dendrites for long periods of time, which serves as a repository of unprocessed antigen (Tew and Mandel, 1979, Tew *et al.*, 1982). FDCs are localised in the central region of primary follicles, in contrast, FDCs in secondary follicles show a polarised distribution. FDCs in the germinal centre light zone display abundant dendrites with a higher level of membrane-bound immune complexes compared to dark zone FDCs, which display fewer dendrites (Allen and Cyster,

2008). Light zone FDCs have been extensively described (Allen and Cyster, 2008) and are associated with upregulated expression of three low affinity Fc receptors, CD23 for IgE and CD16 and CD32 for IgG (Hazenbos *et al.*, 1998, Maeda *et al.*, 1992, Qin *et al.*, 2000) and the integrin ligands inter-cellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and mucosal vascular addressin cell adhesion molecule 1 [MAdCAM-1] (Balogh *et al.*, 2002). In contrast, the properties of dark zone FDCs have not been extensively described, although recently fibrinogen has been shown in association with dark zone FDCs (Lefevre *et al.*, 2007).

The cellular origin of FDCs and the conditions of their development are poorly understood, with early FDC development studies complicated by their resistance to radiation (Kinet-Denoel *et al.*, 1982). Recent studies support the model that FDCs are stromal cells of mesenchymal origin, although it is not certain if the cells originate from within the follicle or migrate from another site (Cyster *et al.*, 2000).

Transplantation experiments in severe combined immunodeficiency (SCID) mice, which lack B cells, T cells and FDCs, have elucidated some of the requirements for FDC development. After reconstitution of SCID mice with donor B cells, FDCs of host origin were observed, suggesting that FDCs developed under the influence of B cells (Yoshida *et al.*, 1995, Yoshida *et al.*, 1994). Similar results were reported for SCID mice reconstituted with bone marrow and fetal liver, however FDCs of host and of donor origin were detected, indicating that progenitor cells were present in the transferred primary lymphoid tissues (Kapasi *et al.*, 1998). Tumour necrosis factor (TNF) and a subset of the TNF-family proteins known as lymphotoxin (LT) are

required for normal FDC development (Cyster *et al.*, 2000). LT can exist either as a secreted protein called LT α 3 which binds the receptors TNFR1 and TNFR2, or as a membrane-bound protein called LT α 1 β 2 which binds the LT β R (Tumanov *et al.*, 2003). LT β R-deficient mice lack FDCs (Allen and Cyster, 2008) and mouse spleens can be depleted of FDCs and retained antigen by administering a LT β R-Ig fusion molecule consisting of the extracellular domain of LT β R and the constant region of human IgG1 (Gatto *et al.*, 2007). In addition, it has been demonstrated that membrane-bound LT on B cells is required for FDC development (Fu and Chaplin, 1999), this would explain the ability of B cells, as described above, to restore FDCs when transferred to lymphocyte deficient mice. It is also important to note that germinal centre B cells have elevated amounts of surface LT α 1 β 2 compared to naïve B cells (Ansel *et al.*, 2000).

Evidence in the literature supporting the stromal derivation of human FDCs is based on studies of cells isolated from tonsils. These cells were CD45 negative, suggesting that they were not bone marrow derived cells. In addition, these cells expressed the α -smooth muscle actin, suggesting that FDCs are a specialised form of myofibroblasts, similar to bone marrow stromal cell progenitors (Munoz-Fernandez *et al.*, 2006, Schriever *et al.*, 1989). It must be remembered that the low proportion of FDCs in lymphoid follicles, together with technical difficulties in their isolation, make these cells very difficult to study. More support for stromal derivation of human FDCs is provided in the literature by evidence of ectopic FDCs associated with conditions of chronic inflammation and rare primary FDC-tumours. These cells have been identified by their expression of the long human isoform of CD21, thought

to be a human FDC-specific molecule (Liu *et al.*, 1997, van Nierop and de Groot, 2002).

1.4.1. Function of follicular dendritic cells

FDCs form an important component of the germinal centre reaction, playing a role in antigen trapping, lymphoid follicle organisation and promoting B cell proliferation, survival and differentiation.

1.4.1.1. Antigen trapping

The ability of FDCs to trap and retain antigen in the form of immune complexes (composed of antibody, complement or antibody and complement) is linked to their variable expression of complement and Fc receptors (CD16, CD23 and CD32) (Hazenbos *et al.*, 1998, Maeda *et al.*, 1992, Qin *et al.*, 2000). The complement receptors CD21 (for complement component 3d) and CD35 (for complement component 3b/4b) are expressed in both primary and secondary follicles (Imal and Yamakawa, 1996) and may play an important role to trap complement containing immune complexes formed rapidly after exposure to a pathogen (Carroll, 1998). Recently, FDCs were identified as the predominant cell type expressing the human Fc receptor for IgA and IgM (Fc α / μ R) (Kikuno *et al.*, 2007). IgM is the first antibody to be produced during a humoral immune response and natural antibodies are mainly IgM (Ochsenbein *et al.*, 1999a), therefore this receptor may play an important role in membrane-bound antigen presentation to B cells during the initial stages of an immune response to a pathogen (Ochsenbein and Zinkernagel, 2000). A number of studies have examined how antigen is presented to B cells in lymph node

follicles using real-time imaging approaches, B cells can encounter soluble antigen that has diffused into the follicle or antigen can be presented by macrophages, DCs or FDCs (Batista and Harwood, 2009, Cinamon *et al.*, 2008, Kraal, 2008, Pape *et al.*, 2007). However, the mechanism of immune complex transport and deposition on FDCs is unknown, future work using high-resolution imaging approaches may provide a better understanding of this important process. Marginal zone B cells in the spleen are able to take up blood-borne antigens, these cells constantly shuttle between the marginal zone and the follicle, carrying antigen to the FDCs (Cinamon *et al.*, 2008, Kraal, 2008).

1.4.1.2. Interaction between B cells and follicular dendritic cells

Antigen, in the form of immune complexes, on FDCs is markedly more effective at stimulating B cell differentiation, proliferation, somatic hypermutation and class switch recombination (Aydar *et al.*, 2005) than soluble antigen or soluble immune complexes (Kosco *et al.*, 1988). The enhanced stimulation is proposed to result from the interaction of B cells with repetitive, membrane-bound antigen on the surface of the FDCs causing clustering of the B-cell receptor and co-receptor complex (Kosco-Vilbois, 2003). However, the importance of the interaction of FDC-bound immune complexes with B cells has been brought into question. In a study of transgenic mice deficient of secreted immunoglobulin, therefore lacking antigen-antibody complexes, there was no effect on germinal centre development or B-cell memory (Anderson *et al.*, 2006). This observation could be explained by FDC-bound complement components interacting with the B-cell co-receptor complex through CD21, providing activation and proliferation signals (Allen and Cyster, 2008). In addition,

the presence of immunoglobulin Fc in antigen-antibody complexes *in vitro* causes inhibition by engagement of the inhibitory FcγRIIB on B cells (Tew *et al.*, 1997). However, it has been proposed that this mechanism could form part of the B-cell selection process (affinity maturation) in germinal centres *in vivo*. B cells with low affinity B-cell receptors may undergo apoptosis as a result of the relatively stronger inhibitory signal received by engagement with FcγRIIB (Ravetch and Nussenzweig, 2007). Furthermore, the high concentration of FcγRIIB on FDCs is thought to bind excess immunoglobulin Fc regions on the immune complexes. This reduces the number of Fc regions available that would otherwise bind FcγRIIB on the B cells, therefore reducing down regulation (Fakher *et al.*, 2001). Another important interaction between membrane-bound antigen and B cells occurs as the germinal centre reaction progresses. As the FDC mature the dendrites form beaded structures coated with immune complexed antigen, these beads are called immune-complex coated bodies or iccosomes (Szakal *et al.*, 1988). These iccosomes are dispersed to B cells (or other antigen presenting cells) where they are endocytosed and processed for MHC class II presentation to T cells (Tew *et al.*, 2001). FDCs can retain antigen for long periods of time and immune complex deposition on FDCs may be involved in maintaining neutralising antibody titres, memory cells and recall responses (Gatto *et al.*, 2007). FDC also provide a number of B-cell trophic factors and cytokines including B-cell activating factor (BAFF), which is able to rescue germinal centre B cells from apoptosis *in vitro*, and membrane bound IL-15 which augments B-cell proliferation (Park and Choi, 2005).

1.4.1.3. Organisational functions

When FDC receive the proper developmental and maturation signals they cluster and express the B-lymphocyte chemokine CXCL13 (which is strongly dependent on LT α 1 β 2 and TNF) for which B cells constitutively express the receptor CXCR5. B cells migrate and home into follicles under the influence of CXCL13 to form the germinal centre (Chaplin and Zindl, 2006). CXCL13 induces LT α 1 β 2 production by B cells providing a positive feedback loop (Ansel *et al.*, 2000). FDCs appear to have higher ICAM-1 expression than any other cell type in the lymph node. The adhesion molecules play a major role in FDC and B-cell interaction mainly via ICAM-1 and VCAM-1 pathways (Koopman *et al.*, 1991, Tew *et al.*, 1997).

1.5. The germinal centre reaction

The HEVs within lymph nodes secrete the chemoattractant cytokine CCL21 (C-C motif chemokine ligand 21) (Hedrick and Zlotnik, 1997). DCs (these cells also express CCL21) and T cells expressing the CCL21 receptor CCR7 (C-C motif chemokine receptor 7) (Yoshida *et al.*, 1997) migrate out of the HEV into the T-cell zone of the lymph node. Recirculating B cells, which also express CCR7, enter the lymph node via the HEV and migrate to the primary follicle under the influence of CXCL13 and CXCL12 (Allen *et al.*, 2004, Chaplin and Zindl, 2006). Entry of cells into the spleen is from the splenic artery, cells migrate to the white pulp in the periarteriolar lymphocyte sheath. Recirculating T and B cells move to the red pulp and exit the spleen in the venous blood (Welsh *et al.*, 2004). Specific B cells are trapped at the border between the follicle and the T-cell zone where they proliferate forming a primary focus after interaction of antigen with the B-cell antigen receptor

(BCR) and after receiving the appropriate costimulatory signals. These proliferating cells will either migrate to extrafollicular areas and differentiate into short lived antibody-producing plasma cells (with an approximate half-life of 3 to 5 days *in vivo*) (Ho *et al.*, 1986) or migrate to the nearby follicle to participate in the germinal centre reaction (MacLennan, 1994).

B cells undergo a number of modifications during the germinal centre reaction. Within the germinal centre dark zone, B cells proliferate and undergo somatic hypermutation, altering the variable regions of their immunoglobulin gene (Kim *et al.*, 1981). During this process the large rapidly proliferating B cells, termed centroblasts, reduce their surface immunoglobulin expression. The process of somatic hypermutation introduces point mutations into the variable regions of the heavy and light chain immunoglobulin genes at a very high rate, giving rise to a large number of mutant BCRs with variable affinity for the antigen (McHeyzer-Williams and McHeyzer-Williams, 2005). As development progresses the B cells move into the FDC-populated light zone of the germinal centre. These small, non-proliferating B cells, termed centrocytes, compete for binding antigen on FDCs and are subjected to the process of positive and negative selection, isotype switching and differentiation (Tarlinton and Smith, 2000). Cells with improved affinity for the antigen are selected and expanded either by the prevention of cell death and/or the enhancement of cell division resulting in isotype switching and differentiation (McHeyzer-Williams and McHeyzer-Williams, 2005). Isotype switching via irreversible DNA recombination enables the assembled high affinity variable gene region, selected after somatic hypermutation, to be expressed on different constant

immunoglobulin chain regions. Switching to other isotypes only occurs after the B cell has been stimulated by antigen. Isotype switching in T-D immune responses requires the interaction between helper T cells and B cells. The CD40L/CD40 interaction between these cells is considered the most important interaction for a sustained and isotype switched immune response to a T-D antigen. Isotype switching can also occur during a T-I immune response with the development of a thymus-independent germinal centre (Gaspal *et al.*, 2006, Zubler, 2001). Recent advances using real-time imaging has shown that the germinal centre reaction is a much more dynamic process, challenging the classical germinal centre model described above (Allen *et al.*, 2007b, Hauser *et al.*, 2007, Schwickert *et al.*, 2007). Germinal centre B cells are actually highly motile and transit in both directions between the germinal centre light and dark zones, a process regulated by the level of CXCR4 receptor expression for CXCL12 expressed on B cells (Allen *et al.*, 2004, Allen *et al.*, 2007a). In addition, dark zone and light zone B-cell morphology has been shown to be similar, with proliferation and cell death occurring in both zones and competition not only for antigen, but also for T-cell help (Allen *et al.*, 2007a, Allen *et al.*, 2007b, Hauser *et al.*, 2007, Schwickert *et al.*, 2007). The T helper cells may also undergo a degree of antigen-driven selection during the germinal centre reaction (Zheng *et al.*, 1996). However, not all T helper cells enter the germinal centre reaction and the germinal centre phase is not thought to be necessary for memory T cell development (Mikszta *et al.*, 1999).

The B cells that survive the germinal centre reaction differentiate into plasmablasts and finally into plasma cells or memory B cells (Tarlinton and Smith, 2000). The

plasma cells migrate to bone marrow niches and potentially live for a long period, obtaining signals from bone marrow stromal cells and continuously producing specific antibody (McHeyzer-Williams and McHeyzer-Williams, 2005). The antigen specific memory B cells do not secrete antibody, but constantly migrate between the blood circulation and tissues, able to respond rapidly when re-exposed to antigen to provide an enhanced adaptive immune response (Good *et al.*, 2009).

1.6. Maintaining immunity

Immunological memory is the ability of the adaptive arm of the immune system to recognise and respond more rapidly to an antigen that it has encountered previously with a robust immune response to protect the host from re-infection, control persistent infections and to protect offspring from primary infection (Ahmed and Gray, 1996). Adaptive immunity, which is responsible for immunological memory, can be broadly divided into two linked compartments, humoral immunity, consisting of circulating antibody and the cells involved in their production, or cell mediated immunity to kill infected cells (Zinkernagel, 1996).

1.6.1. Maintaining cellular immunity

The $\alpha\beta$ T cells play an essential role in maintaining immunological memory. The frequency of T cells that recognise a specific peptide antigen is usually low, with lymphocyte circulation increasing the chance of these encounters (Selin *et al.*, 1994). On contact with peptide presented on MHC molecules on the surface of antigen presenting cells, the specific T-cells proliferate and differentiate generating a large number of effector cells that migrate to tissues to help eliminate the specific

pathogen. During the contraction phase of the immune response there is a general migration of spleen and lymph node T cells to peripheral tissue, a process referred to as “diaspora” (Marshall *et al.*, 2001), during which a large number of activated T cells undergo apoptosis (Razvi *et al.*, 1995). Some of the primed T cells do not undergo apoptosis but develop into either “effector” or “central” memory T cells. Compared to naïve T cells, the memory T cells have a higher affinity for the specific peptides (Welsh *et al.*, 2004). In addition, downstream signalling on TCR engagement is enhanced leading to more rapid induction of effector functions compared to naïve T cells (Kersh *et al.*, 2003, Slifka *et al.*, 1999). The “effector” memory T cells lack lymph node homing receptors (CCR7^{low}) but express receptors for homing into inflamed tissue (Sallusto *et al.*, 1999). Upon re-encounter with antigen they produce chemokines and cytokines, for example, IFN γ or IL-4 (CD4⁺ cells) or release stored cytotoxic factors, for example perforin, in the case of CD8⁺ memory T cells (Sprent and Surh, 2002). The “central” memory T cells express lymph node homing receptors (CCR7^{high}). These cells have a lower activation threshold and cycle more rapidly than “effector” memory T cells (Sallusto *et al.*, 1999, Zinkernagel *et al.*, 1996). Upon re-encounter with antigen they proliferate and differentiate into effector cells, migrate into peripheral tissue and mediate effector functions (Welsh *et al.*, 2004). It is not clear how the pool of high frequency memory T cells specific for a single peptide are maintained and whether this pool can be maintained in the absence of specific antigen stimulation (Lau *et al.*, 1994).

Lymphocytic choriomeningitis virus (LCMV) infection is non-cytopathic in mice and initial control is largely dependent on a cytotoxic T-lymphocyte response, as opposed to neutralising antibody (Bachmann and Zinkernagel, 1997, Fehr *et al.*,

1996, Lee *et al.*, 2005). The data from mouse adoptive LCMV immune-cell transfer studies in the literature seem contradictory. There is evidence for the requirement of persisting viral antigen in order to maintain the antiviral protective capacity of the transferred cells (Gray and Matzinger, 1991, Oehen *et al.*, 1992), whereas other investigators have reported that cytotoxic T lymphocytes persist and maintain protective immunity against challenge for up to 2 years in the absence of antigen (Lau *et al.*, 1994). Evidence of persisting T-cell memory in humans following immunisation with vaccinia virus during childhood seems to support the hypothesis that continuous specific antigenic stimulation is not required, however, these studies do not demonstrate the absence of persisting antigen (Sprent and Surh, 2002). *In vitro* stimulation assays have identified specific CD4⁺ and CD8⁺ memory T cells up to 50 years after immunisation and virus-specific CD4⁺ T cells have been identified in smallpox vaccinated individuals with a half-life up to 12 years (Amara *et al.*, 2004, Crotty *et al.*, 2003, Demkowicz *et al.*, 1996). It is not clear if the detected responses are protective. Booster immunisation was recommended every 10 years to maintain vaccine efficacy. In addition, persisting memory B cells are also able to mount a robust anamnestic antibody response, with no correlation between stable antibody titres and T-cell memory (Crotty *et al.*, 2003, Hammarlund *et al.*, 2005). However, evidence from a vaccine trial testing a recombinant vaccinia virus expressing HIV gp160 identified poor responders on the basis of the long lived T-cell-memory response following smallpox vaccination compared to vaccinia virus naïve individuals, suggesting that the long lived T-cell-memory response is protective (Cooney *et al.*, 1991). An additional complication for maintaining an effective pool of memory T cells under field conditions is the continuous competing

immune challenges which the immune system is subjected to. Deletion of pre-existing memory T cells occurs during virus induced lymphopaenia (McNally *et al.*, 2001) and during heterologous viral and bacterial infections (Selin *et al.*, 1996, Smith *et al.*, 2002), circumstances during which persisting antigen may be beneficial.

1.6.2. Maintaining humoral immunity

Serum antibodies are a critical component for protection against FMDV and there is a close correlation between protection from disease after recovery from infection or after immunisation and the titre of circulating antibodies (Alexandersen *et al.*, 2003b). FMDV infection in ruminants elicits an immune response that can provide protection for several years (Cunliffe, 1964). Similarly, humoral immunity to viral infections can last for decades in humans and for the lifetime of mice (Slifka and Ahmed, 1996). As serum antibodies have a short half-life (Talbot and Buchmeier, 1987), reported to be less than 3 weeks in adult mice (Vieira and Rajewsky, 1988), continual replenishment either by long-lived plasma cells, activation of memory B cells to differentiate into plasma cells or ongoing recruitment and differentiation of naïve B cells into antibody secreting plasma blasts and plasma cells is required to maintain stable long-term protective humoral immunity (Wrammert and Ahmed, 2008).

As discussed under section 1.5, production of long lived plasma cells and memory B cells is dependent on the germinal centre reaction. The migration of antibody secreting cells from lymphoid organs to peripheral tissue, including the bone marrow, is regulated by the expression of adhesion molecules and chemokine

receptors. However, the tissue specificity of the adhesion molecules and the mechanisms governing recruitment are still not clear (Manz *et al.*, 2005). The chemokine receptor CXCR4 has been identified as an important receptor for plasma blast migration to bone marrow, attracted to its ligand CXCL12 expressed on bone marrow stromal cells (Hargreaves *et al.*, 2001). The plasma blasts can differentiate and persist as long-lived plasma cells by competing with established plasma cells for a limited number of “plasma cell survival niches” (Odendahl *et al.*, 2005, Tokoyoda *et al.*, 2004). Such niches are found predominantly in bone marrow although additional niches exist in organised lymphoid tissue, for example, the spleen and in inflamed tissue (Manz *et al.*, 2005). Recently, the molecular basis of bone marrow B-cell survival niches has begun to emerge, with bone marrow stromal cells and bone marrow-resident DCs playing a critical role (Manz *et al.*, 2005, Sapoznikov *et al.*, 2008). The reticular cells which surround the vascular sinuses (called CAR cells) express the chemokine CXCL12 on their long processes, plasma cells express CXCR4 and respond by improved survival (Cassese *et al.*, 2003, Hargreaves *et al.*, 2001, Tokoyoda *et al.*, 2004). B-cell maturation antigen (BCMA) expressed on plasma cells and its ligands BAFF and a proliferation activation ligand (APRIL) have also been identified as important plasma cell survival factors (Manz *et al.*, 2005). Perivascular clusters of bone-marrow resident DCs promote survival of recirculating mature B cells through production of macrophage migration-inhibitory factor (MIF) (Sapoznikov *et al.*, 2008). Interaction with its receptor CD74-CD44 on B cells triggers an antiapoptotic signalling pathway thus promoting B-cell survival (Leng *et al.*, 2003). These bone-marrow resident DCs have also been shown to produce BAFF and APRIL (Sapoznikov *et al.*, 2008).

Memory B cells leave the germinal centre reaction by an unknown mechanism and enter the recirculating memory B-cell compartment (McHeyzer-Williams and McHeyzer-Williams, 2005). Memory B cells are in a resting state, able to persist in the absence of both cell division and signal through the B-cell receptor, and only secrete antibody when antigenically stimulated or by polyclonal activation (Bernasconi *et al.*, 2002, Maruyama *et al.*, 2000). Memory B cells have been detected in cattle by enzyme-linked immunosorbent spot (ELISPOT) assay as cells that secrete antibody after *in vitro* antigen restimulation (Lefevre *et al.*, 2009). Memory B cells have increased expression of TNF receptor families and TLR-related molecules compared to naïve B cells, subsequently they exhibit enhanced survival, enhanced antibody secretion and enter cell division more rapidly than naïve B cells (Good *et al.*, 2009). In addition, they have an enhanced ability to stimulate T cells by expressing CD80 and CD86 which interact with CD152 expressed on activated T cells (Good *et al.*, 2009, Vasu *et al.*, 2003).

Analogous to maintaining T cell memory, the requirement of persisting antigen to maintain humoral immunity remains debated. Adoptive transfer studies have clearly demonstrated in mice that in hosts with relatively short lifespans, specific antibody is continuously replenished by long-lived plasma cells in the absence of memory B cells and antigen (Manz *et al.*, 1997, Slifka *et al.*, 1998). However, uncertainty exists of the ability of plasma cells alone to maintain protective titres of neutralising antibody under conditions of serial infections in animals with longer lifespans (Welsh *et al.*, 2004). Persisting antigen, in the form of immune complexes attached

to FDCs can provide signal through the B-cell receptor to induce memory B-cell proliferation and differentiation into plasma cells for maintaining protective titres of antibody (Bachmann and Zinkernagel, 1997, Ochsenbein *et al.*, 2000b). In addition, antigen trapped on FDCs can induce naïve B-cell proliferation and differentiation into plasma blasts and memory B cells, therefore persisting FDC-bound antigen can also play an important role in maintaining humoral immunity by repopulating the memory B cell pool (Gray and Skarvall, 1988, Kosco-Vilbois, 2003). This hypothesis is particularly relevant for the situation in the field because it is not clear how the memory B cell pool is restored and maintained after repeated engagement with antigen (Welsh *et al.*, 2004). However, antigen-antibody complexes on FDCs are reported to have a relatively short half-life of approximately 8 weeks (Tew and Mandel, 1979) suggesting this mechanism is not required for sustaining lifelong immunity, for example, following smallpox vaccination in humans where antibody titres remain nearly constant for up to 75 years after immunisation (Crotty *et al.*, 2003, Hammarlund *et al.*, 2003). Indeed, late antigen dependent germinal centres, which are still detectable up to 100 days after immunisation (Bachmann *et al.*, 1996), are not required to maintain antibody titres or B cell memory (Gatto *et al.*, 2007). These investigators suggested that the late germinal centre reaction may be important for maintaining a flexible, hypermutated B cell repertoire in case of pathogen re-emergence (Gatto *et al.*, 2007). Elimination of sequestered antigen on FDCs by injection of LT β R-Ig fusion proteins on days 9 to 11 post immunisation had a detrimental effect on antibody titres in mice, highlighting the importance of persisting antigen during the early phase of the B-cell response when germinal centres are producing large numbers of plasma and memory B cell precursors (Gatto

et al., 2007). These investigators also reported that bone marrow plasma cells do not survive for the lifetime of the mouse but decline with a half-life of 3 months (Gatto *et al.*, 2007). A similarly short plasma cell half-life of approximately 140 days has also been reported in mice depleted of memory B cells by irradiation (Slifka *et al.*, 1998) highlighting the importance of the size of the memory B-cell compartment and memory B-cell survival for maintaining long-term and effective humoral immunity (Dörner and Radbruch, 2007, Gatto *et al.*, 2007). An alternative mechanism to replenish plasma cells and subsequently maintain neutralising antibody titres has been described which involves polyclonal stimulation to sustain memory B-cell proliferation and differentiation in the absence of antigen (Bernasconi *et al.*, 2002). Memory B-cell differentiation into antibody producing cells can be induced by microbial products, for example, lipopolysaccharides via TLR4 and unmethylated single-stranded DNA motifs via TLR9. In addition, T cell activation by third party antigens can stimulate B cells via CD40/CD40L and in contrast to naïve B cells, the cytokine IL-15 can trigger memory B-cell activation in the absence of antigen (Bernasconi *et al.*, 2002). The mechanisms by which memory B cells and long-lived humoral immunity is maintained remain unclear and are currently active fields of research, however it is clear that FDC-bound antigen is pivotal to the germinal centre reaction, playing an important role in maintaining humoral immunity.

2. FMDV persists in the light zone of germinal centres

2.1. Introduction

The paucity of our understanding of the mechanisms underlying FMDV persistence and the short term duration of protection after vaccination, which contrasts with the prolonged duration of immunity after natural infection, are major factors hindering global FMDV control policies. Virus is cleared rapidly from blood during the acute stage of FMD, coinciding closely with the emergence of an antiviral antibody response characterised by high-affinity circulating neutralising antibodies, a crucial component of the immune response against FMDV (Alexandersen *et al.*, 2003b). This is in contrast to pharyngeal tissue including the soft palate, nasopharynx, oropharynx, palatine tonsil and mandibular lymph node, which, despite the high titres of circulating virus neutralising antibody, have been shown to contain viral RNA for up to 72 days after infection (Zhang and Alexandersen, 2004). The significance of continued detection of viral RNA has not been clear since FMDV proteins have not been detected, in previous studies in these tissues, following the resolution of vesicular lesions. Importantly, FMDV proteins have not been detected previously in lymphoid tissue *in vivo* at any stage of infection and viral proteins have not been detected in any tissue following resolution of vesicular lesions.

A number of different pathologically relevant proteins, organisms and their products have been shown to be retained on FDCs in lymphoid tissue, for example, human, feline and simian immunodeficiency virus (Tenner-RÁCz *et al.*, 1985, Toyosaki *et al.*,

1993, Ward *et al.*, 1987), the pestivirus bovine viral diarrhoea virus (Fray *et al.*, 2000), murine leukaemia virus (Hanna *et al.*, 1970, Siegler *et al.*, 1973, Szakal and Hanna, 1968), VSV (Bachmann *et al.*, 1996), tetanus (Kosco-Vilbois, 2003) and disease-associated prion proteins (McGovern and Jeffrey, 2007). The ability of FDCs to trap and retain antigen and infectious virus in a stable conformational state in the form of immune complexes for months or even years within germinal centres and their intimate association with B cells is a crucial component of the humoral response (Haberman and Shlomchik, 2003). FDCs are important for the development of follicles during the early immune response, B cell affinity maturation and memory B cell development either through the presentation of surface-retained antigen to B cells or by supporting B-cell proliferation and differentiation in a non specific manner (Haberman and Shlomchik, 2003, Kikuno *et al.*, 2007). Additionally, the slow release of antigen from the surface of FDCs is thought to play a role in maintaining serum titres of specific antibody and studies have shown that the amount of retained antigen can regulate serum immunoglobulin titres (Szakal *et al.*, 1992, Szakal *et al.*, 1989, Tew *et al.*, 1980).

2.1.1. The FMDV ‘carrier’ problem

Over 50% of ruminants exposed to viral challenge, whether vaccinated or not, can become ‘carriers’ (Alexandersen *et al.*, 2003b). It is not a lifelong infection with species and viral strain variation, for example, there are reports of individual cattle carrying virus for up to 3.5 years (Hedger, 1968), 9 months in sheep and goats (Burrows, 1968) and at least 5 years in Africa buffalo (Condy *et al.*, 1985, Thomson *et al.*, 2003). Pigs normally clear virus from oropharyngeal fluid within 3 weeks of

infection and are not considered to be involved in 'the carrier problem'. However, viral RNA has been detected in cervical lymph nodes, mandibular lymph nodes and tonsils of pigs at 28 days post-infection (Zhang and Bashiruddin). Recovery of infectious virus from oropharyngeal scrapings of FMD recovered ruminants is intermittent and the titre of virus recovered from 'carrier' animals is low, often falling below the titre thought to be necessary for successful transmission to susceptible animals (Donaldson and Kitching, 1989). Intermittent virus recovery may be related to the heterogeneous nature of oropharyngeal samples with saliva, mucus and cells present in varying quantities (Alexandersen *et al.*, 2002). In addition, the virus is thought to be associated with cellular material and Freon treatment, to remove blocking antibodies and cellular membranes, can increase viral titres by several orders of magnitude (Brown and Cartwright, 1960).

2.1.1.1. Evidence of transmission from 'carrier' animals

'Carrier' African buffalo have been shown to be a source of infection for other susceptible species with variable transmission from 'carrier' buffalo to cattle reported under experimental conditions (Bastos *et al.*, 2000, Vosloo *et al.*, 2002). This is in contrast to the unknown epidemiological significance of 'carrier' cattle. Transmission from 'carrier' cattle has not been demonstrated under experimental conditions, even under conditions of co-infection with rinderpest and bovine herpes 1 viruses (McVicar, 1977). In one series of experiments, 'carrier' cattle were treated with dexamethasone in order to depress their immune systems, and kept in contact with susceptible cattle, but this had the reverse effect of causing the virus to disappear from oropharyngeal scrapings, only to reappear once the treatment was

stopped (Ilott *et al.*, 1997). There was no transmission between ‘carrier’ and susceptible cattle. Despite the uncertainty concerning the capacity of ‘carrier’ cattle to transmit virus, there is a requirement to identify and remove these animals before a country or region can declare freedom from infection and resume international animal trade.

2.1.1.2. Sites and proposed mechanisms of FMDV persistence

In situ hybridization studies have supported the generally accepted hypothesis that FMDV persists in the epithelium of the dorsal soft palate and oropharynx dorsal to the soft palate in cattle (Alexandersen *et al.*, 2002). These studies identified FMDV RNA associated with epithelial cells in the stratum germinativum, but not in the more superficial epithelial layers of the dorsal soft palate, up to 82 days post-infection (Prato Murphy *et al.*, 1999, Zhang and Kitching, 2001). However, viral proteins have not been identified in association with this tissue even during the acute stage of FMD and the mechanism of persistence at this site is not clear. In addition, it is unclear how the virus is excreted into the pharynx or detected by probang sampling at these sites.

Various mechanisms have been proposed for the development of FMDV persistence, most of the mechanisms described are based on immune evasion strategies that are employed by other viruses to establish and maintain persistence. In order for highly cytopathic viruses like FMDV to establish persistent infections, they must have mechanisms to moderate their replication and to escape the host immune response either through evasion or direct suppression (Borrow *et al.*, 1991). It is clear that

FMDV is efficient at establishing persistent infections in ruminants, and that FMDV is highly immunogenic and does not induce an ineffective immune response in ‘carrier’ animals. Immunity to FMDV is primarily mediated by neutralising antibody and there is no consistent failure or deficiency in the antibody response of animals that become persistently infected. Indeed, local and systemic antibody responses are prolonged in ‘carrier’ animals and it has been shown in FMD convalescent cattle that resistance to re-infection and local virus replication in the oropharynx shows a strong correlation with a history of persistent infection (McVicar and Suttmoller, 1974, Salt, 1993, Salt *et al.*, 1996a).

Some viruses are known to persist by residing in “immunologically privileged” sites. These sites, which include for example, the eye and central nervous system, are characterised by active and passive processes which result in the survival of allografts that would otherwise be promptly rejected if placed at other body sites (Streilein, 1993). Theiler’s murine encephalomyelitis virus (TMEV), a picornavirus in the genus *Cardiovirus*, is a neurotropic virus that takes advantage of immune privilege and induces a persistent central nervous system infection in mice (Ghadge *et al.*, 1998, Ricour *et al.*, 2009). An additional example is herpes simplex virus which establishes a latent infection in neurons, taking advantage of the fact that neurons do not express MHC class I, thereby avoiding a cytotoxic T-cell response (Banks and Rouse, 1992). The epithelium of the dorsal soft palate and adjacent oropharynx in the ruminant have been proposed to act as “immunologically privileged” sites, able to support FMDV replication and evade serum antibody (Alexandersen *et al.*, 2002, Salt, 2004).

Viruses can also interfere with the host immune response, to suppress or induce an ineffective response and establish persistence. Interference can be caused by active infection of cellular components of the immune system, for example, Epstein-Barr virus, poliovirus and bovine viral disease virus can establish persistent infections in lymphocytes (Deregt and Loewen, 1995, van Loon *et al.*, 1979, Young and Rickinson, 2004). FMDV can infect antigen presenting cells of a number of different species *in vitro*, infection of bovine monocyte-derived DCs *in vitro* has been shown to result in cell death and as a consequence, the amount of antigen processed and presented by the DCs to T cells is reduced (see sections 1.3.1.4 and 1.3.1.5). Infection and impairment of the function of this important antigen presenting cell type *in vivo* may influence elimination of the virus. Interference can also be mediated by a number of different virally encoded immune modulators that are capable of prejudicing antigen presentation, cytokine function and apoptosis to aid host immune evasion (Spriggs, 1996). Viral proteins that regulate antigen presentation can interfere with the cellular immune response to prevent destruction by NK cells and cytotoxic T cells. MHC class I expression is known to be down-regulated on FMDV infected epithelial cells (Sanz-Parra *et al.*, 1998). FMDV is highly cytotoxic and analogous to other lytic viruses, infection can result in decreased surface MHC expression simply as a result of overall shut-off of host protein synthesis, this strategy may diminish the cytotoxic T-cell response, however it does not preserve the cell for persistence. FMDV 2BC protein has been shown to block transport of proteins through the ER-Golgi pathway (Belsham, 2005, Moffat *et al.*, 2005). The ER and Golgi apparatus are important for the delivery of proteins to the surface of cells and poliovirus 3A protein, which also blocks this pathway, has been shown to

reduce the secretion of cytokines, for example, type I IFN, IL-6 and IL-8 and to compromise MHC class I presentation (Dodd *et al.*, 2001). In addition to the example of poliovirus, a number of other lytic viruses that are known to persist, for example herpesviruses and adenoviruses, have developed similar subtle strategies to shut off MHC class I expression (Spriggs, 1996) and analogous to these viruses, FMDV would require additional mechanisms to moderate replication to preserve the host cell for persistence.

Another proposed mechanism of FMDV persistence *in vivo* is viral attenuation in order to reduce cytolysis of the infected cells (Salt, 1993, Straver *et al.*, 1970). “Persistently infected” cell cultures have been established for FMDV (de la Torre *et al.*, 1985, Herrera *et al.*, 2008). These cells maintained FMDV RNA with multiple genetic variations and large deletions in association with the expression of viral proteins, but did not maintain infectious virus. These results should be interpreted with caution in relation to the situation *in vivo* as the persistent infection was established in a genetically unstable Syrian hamster tumour cell line and the perceived attenuation may be the result of selection of cellular phenotypes with increased resistance to FMDV (Martin Hernandez *et al.*, 1994, Stoker and MacPherson, 1964). J. Salt (2004) suggested that the co-evolution of FMDV with resistant cells reflected in these *in vitro* infection models may occur *in vivo* between the dividing basal layer cells of the pharyngeal epithelium and persisting FMDV. Naturally lytic viruses may also regulate their gene expression to reduce cytolysis and interfere with cell metabolism to provide intracellular conditions favourable for long term persistence. Latent infections are defined as persistent viral infections of

cells in which the viral genome is present, but gene expression is limited and infectious virus is not produced (Banks and Rouse, 1992). Latency is best demonstrated by the herpesviruses as a strategy to persist and evade immune surveillance. There are reports in the literature describing a “latent” form of infection with two members of the *Picornaviridae* family, coxsackieviruses B1 and B2 (Cunningham *et al.*, 1990, Tam *et al.*, 1991). A role for this method of persistence during FMD has not been described (Salt, 2004).

RNA viruses are characterised by a high degree of variation and a high mutation rate, subsequently, the genome of FMDV and of other RNA viruses is highly unstable (Domingo *et al.*, 2003, Holland *et al.*, 1982). Mutations in the viral genome can lead to alterations in surface antigens with subsequent antigenic drift permitting escape from immune control. Antigenic variation can be effective for persistence at the population level and at the individual level. The best-example of antigenic drift at the population level is influenza virus where mutations in the hemagglutination and/or the neuramidase glycoproteins lead to sequential epidemics in the population. The best-example at the individual level are lentiviruses, for example equine infectious anemia and maedi-visna virus (Clements *et al.*, 1988). Similar to other retroviruses, the lentiviruses use genomic integration of proviral DNA as a mechanism of persistence, however these viruses target end stage cells of the monocyte-macrophage lineage (Narayan *et al.*, 1982) and must replicate and disseminate to other target cells for life-long persistence (Narayan *et al.*, 1982). This mechanism is pronounced in the example of equine infectious anemia by sequential episodes of acute haemolytic crises that are not neutralised by pre-existing antibody (Clements *et*

al., 1988). FMDV is not detected in the circulation during persistence in cattle and recurring episodes of disease are not observed, however, considerable genetic and antigenic variation has been detected during persistence *in vivo* and a myriad of different antigenic isotypes of FMDV exist in the field (Cottam *et al.*, 2008, Malirat *et al.*, 1994, Vosloo *et al.*, 1996). It has been suggested that antigenic drift *in vivo* under immune pressure can result in the establishment of a new virus population (Domingo *et al.*, 1989). However, viral populations tend to fluctuate during persistence rather than evolving as a distinct genomic lineage with conserved changes (Malirat *et al.*, 1994). These authors also demonstrated that homologous post-vaccinal serum consistently neutralised all of the FMDV isolates collected throughout the period of persistence. These results have been confirmed by other investigators (Salt *et al.*, 1996b) suggesting that antigenic variation may not be a means of humoral immune evasion or required to maintain persistence at the individual level. In addition, passage of FMDV in cell culture also results in amino acid substitutions and alterations in viral antigenicity in the absence of selective immunological pressure (Rowlands *et al.*, 1983).

2.2. Aims of the chapter

To determine if FMDV is maintained in lymphoid tissue as immune complexes in association with FDCs after acute FMD. This was investigated by:

- ◇ describing the morphological characteristics of the organised lymphoid tissue in the oropharynx of cattle
- ◇ developing enhanced laser capture microdissection techniques in combination with quantitative real time reverse transcription polymerase chain reaction to determine FMDV genome localisation and genome quantities after acute FMD
- ◇ developing sensitive *in situ* hybridization techniques with appropriate controls to corroborate the laser capture microdissection data
- ◇ describing FMDV protein localisation after acute FMD by confocal microscopy using existing MAbs directed against non-structural proteins and selected anti-capsid MAbs able to detect FMDV immune complexes
- ◇ attempting to isolate viable virus from lymphoid tissue from 29 days post-infection using existing virus isolation techniques and new techniques to dissociate virus from tissue and to detect immune complexed virus

2.3. Materials and methods

2.3.1. Experimental procedures

Animal experiments were carried out at the Institute for Animal Health, Pirbright, in biosecure animal isolation units, under project licence PPL70/6212 in accordance

with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

2.3.1.1. Virus inoculation

The virus strains used for inoculation were FMDV O UKG 34/2001 and O1 BFS 1860. The original suspension of O UKG 34/2001 was obtained from a pig at Cheale Meats Abattoir, Brentwood, Essex (WRL 17.4.01). This material was used to intradermolingual challenge 2 cattle UI94 and UI95. The material used for subsequent inoculations was ground up vesicular epithelium from these 2 cattle diluted in M25-phosphate buffer (Appendix 1). 0.2mL of the O UKG 34/2001 inoculum was administered subepidermo-lingually to donor animals to deliver a challenge of approximately 10^5 tissue culture infectious dose (TCID)₅₀ (as measured by virus titration on bovine thyroid cells) (Snowdon, 1966). These infected donor animals were subsequently used to infect other cattle by direct contact challenge. FMDV O1 BFS 1860 was provided by T Jackson, IAH. 0.5mL of the original O1 BFS 1860 BTY tissue culture supernatant was administered subepidermo-lingually to donor animals to deliver a challenge of approximately 5×10^5 TCID₅₀ (Snowdon, 1966). These infected donor animals were subsequently used to infect other cattle by direct contact challenge.

2.3.1.2. Sample collection

Killing of animals was carried out by intravenous administration of pentobarbitone (Vetoquinol, France).

Oropharyngeal scrapings were collected at post-mortem using probang sampling cups, split into aliquots and stored at -80°C (Alexandersen *et al.*, 2002). Tissue samples were harvested at post-mortem from infected and non-infected control animals. Fresh instruments and gloves, RNaseZap (Ambion, UK) and 70% v/v ethanol (VWR International, UK) diluted in nuclease-free water (Ambion, UK) were used between tissues and animals to reduce contamination. Portions of the tissue were placed into Peel-A-Away Molds (Thermo Electron Corporation, USA) containing cryomatrix (Sakura Finetek, NL) and frozen on dry ice. These samples were stored at -80°C for immunohistochemistry, *in situ* hybridization and laser capture microdissection. Portions of the tissue were placed into 2mL screw cap micro tubes containing 1mL ($10 \times$ volume) of RNAlater (Ambion, UK). These samples were stored at 2 to 8°C overnight then moved to storage at -80°C for RNA extraction. Portions of the tissue were placed into 7mL glass bijoux tubes containing 50% v/v glycerol (VWR International, UK) in M25-phosphate buffer (Appendix 1) and stored at -20°C for virus isolation. Portions of the tissue were placed into 4% w/v paraformaldehyde (Sigma-Aldrich, UK) in phosphate buffered saline (PBS) [central services unit (CSU), IAH], stored overnight at 2 to 8°C then transferred to 1% v/v paraformaldehyde in PBS for paraffin embedding and hematoxylin and eosin (H&E) staining (kindly performed by H Eburne, IAH).

2.3.2. Enhanced laser capture microdissection technique

The membrane-based laser capture microdissection (LCM) protocol was adapted from a protocol described previously (Allen *et al.*, 2004). Approximately $7\mu\text{m}$ thick, cryosections were affixed to RNase-free steel framed PET-membrane slides (Leica,

UK). The slides were dried for 10 minutes then fixed in 100% cold ethanol (VWR International, UK) for 20 seconds. The slides were dried for 5 minutes then stained in 0.25µm filtered 1% w/v toluidine-blue (Sigma-Aldrich, UK) in nuclease-free water (Ambion, UK) for 3 minutes. Slides were rinsed twice in nuclease-free water for 15 seconds and once in 75% v/v ethanol in nuclease-free water. Slides were dehydrated in 100% ethanol, air dried for 5 minutes and transferred immediately to the stage of the Leica AS LMD (Leica, Germany) for microdissection. Microdissected tissue sections were collected into the caps of 0.2mL RNase-free PCR tubes (Ambion, UK) containing 75µL of lysis buffer RLT (RNeasy Micro Kit; Qiagen, UK). Samples were vortexed for 30 minutes and stored at –80°C until processing. RNA was isolated from the samples with the RNeasy Micro Kit with ‘one column’ DNase treatment (Qiagen, UK), eluted with 15µL nuclease free water, divided into aliquots and stored at –80°C until processing. Twelve µL of the RNA was used for quantitative real-time reverse transcription polymerase chain reaction (rRT-PCR), 1µL of the RNA was used for total RNA quantification (NanoDrop ND-1000 photospectrometer; Thermo Scientific, USA).

2.3.3. Synthesis of bovine 28s rRNA standards

2.3.3.1. RNA extraction and reverse transcription

Heparinised peripheral blood was collected from a conventionally reared and housed British Holstein Friesian. The blood was diluted 1:2 with PBSa (Invitrogen, UK). 35mL of diluted blood was underlayered with 14mL Histopaque-1077 (Sigma-Aldrich, UK) before centrifugation at 1000×g, for 30 minutes at 18°C with the centrifuge brake off. Cells at the interface were collected and washed by dilution in chilled

PBSa and centrifugation at 600×g for 10 minutes at 8°C. Cells were resuspended in 5mL red blood cells lyses buffer (Appendix 1) and held on ice for 5 minutes. Second and third washes were carried out by dilution in PBSa and centrifugation at 250×g for 8 minutes at 8°C. PBMC were counted and total RNA extracted using TRIzol Reagent (section 2.3.5.1). Purified total RNA was reverse transcribed using TaqMan Reverse Transcription Reagents (section 2.3.6.1).

2.3.3.2. PCR amplification, digestion and ligation into pGEM-11Zf(+) vector

Amplification of DNA was performed using *Pfu* DNA polymerase (Stratagene, UK). Each 100µL reaction mix contained 200ng of genomic DNA template (NanoDrop ND-1000 photospectrometer, Thermo Scientific, USA) and 0.5µM forward and reverse primers 28sF and 28sR (Appendix 2) containing restriction enzyme recognition sites for *EcoRI* and *BamHI* respectively at the 5' prime ends. The samples were denatured at 94°C for 45 seconds, annealed at 55°C for 45 seconds and extended at 72°C for 1 min during 30 cycles in accordance with Stratagene's suggested cycling parameters. The PCR product was analysed on a 1% agarose gel (Appendix 1). After gel purification (Qiaprep Gel Extraction Kit; Qiagen, UK) and quantification the product and pGEM-11Zf(+) vector were digested with restriction enzymes *EcoRI* and *BamHI* (section 2.3.8). The digested products were analysed on a 1% agarose gel, purified, quantified and ligated using T4 DNA Ligase (Promega, UK). The vector was then transformed (section 2.3.9) into competent DH5a *E. coli* cells (kindly provided by J Seago, IAH).

2.3.3.3. Sequencing, transcription, purification and quantification

Plasmid DNA containing the 261 base pair PCR product was extracted from overnight DH5 α *E. coli* cell cultures (section 2.3.9) using Qiaprep Spin Miniprep Kits (Qiagen, UK). Sequencing (section 2.3.7) was performed to ensure that the insert contained the correct sequence in the correct orientation. The extracted plasmid DNA was linearised by restriction enzyme digestion (section 2.3.8) with *Bam*HI (Promega, UK). The linearised DNA product was extracted from the digestion reaction using phenol/chloroform/isoamyl alcohol (25:24:21, v/v) and concentration by ethanol precipitation (section 2.3.5.3). The purified, linearised DNA was analysed on a 1% agarose gel to confirm cleavage (Appendix 1), quantified (NanoDrop ND-1000 photospectrometer; Thermo Scientific, USA) and diluted in nuclease free water to a concentration of 0.5 μ g/ μ L in preparation for transcription. A MEGAscript T7 kit (Ambion, USA) incorporating high nucleotide concentrations was used for *in vitro* transcription to ensure ultra-high yield. Each 20 μ L reaction contained 2 μ L T7 RNA polymerase mix, 1 μ g linear DNA, 2 μ L 10 \times reaction buffer, 7.5mM of each ATP, CTP, GTP and UTP solution and nuclease free water. Since the expected 295 nucleotide RNA transcript was significantly shorter than the 500 nucleotide transcript recommended by the kit manufacturers, the reaction was modified for optimal transcription by increasing the incubation time to 6 hours at 37°C. The reaction mix was then treated with TURBO DNase twice at 37°C for 30 minutes and purified with DNase inactivation reagent (TURBO DNase Treatment and Removal Reagents, Ambion, UK). The purity of the single stranded RNA product was estimated by the ratio between the spectrophotometric readings at 260nm and 280nm on a NanoDrop ND-1000 photospectrometer (Thermo Scientific,

USA). The reading at 260nm allowed calculation of the concentration of nucleic acid with an optical density (OD) of 1 corresponding to approximately 40µg/mL single-stranded RNA (Sambrook and Russel, 2001). The molecular weight of the entire 295 nucleotide product was calculated and number of copies/mL determined according to the formula: copies = $(6.023 \times 10^{23} \times \text{g/mL of RNA})/(\text{RNA MW})$ (Yin *et al.*, 2001). A ten-fold dilution series of RNA (nuclease free water; Ambion, UK) was aliquoted into small volumes and stored at -80°C until needed.

2.3.4. Synthesis of FMDV RNA standards

FMDV RNA standards were synthesised *in vitro* from a plasmid (pT7Blue; Novagen, USA) containing a 500 base pair insert of the internal ribosomal entry site of FMDV O UKG 34/2001 (kindly provided by J Horsington, IAH). The enzyme *Bgl* II was used to linearise the plasmid (section 2.3.8). *In vitro* transcribed FMDV RNA standards were prepared as described for 28s RNA under section 2.3.3.3.

2.3.5. Nucleic acid extraction and purification techniques

2.3.5.1. RNA extraction using TRIzol Reagent

Total RNA was isolated with TRIzol Reagent (Invitrogen, UK) using a single-step RNA isolation protocol prescribed by Invitrogen (Chomczynski and Sacchi, 1987). Samples were added to TRIzol Reagent at a volume ratio of 1:3 using at least 0.75mL TRIzol Reagent per 5×10^6 to 10×10^6 cells. The homogenised samples were incubated for 5 minutes at 15 to 30°C to allow dissociation of nucleoprotein complexes. 0.2mL of chloroform (Sigma-Aldrich, UK) was added to the homogenate per 0.75mL TRIzol Reagent. The homogenate was vortexed for 10-15 seconds and

centrifuged at $12000 \times g$ for 15 minutes at 2 to 8°C to separate the mixture into a lower red, phenol-chloroform phase, an organic interphase containing DNA and protein and a colourless upper aqueous phase containing RNA. The aqueous phase was removed and mixed with 0.5mL isopropyl alcohol (Sigma-Aldrich, UK) per 0.75mL TRIzol Reagent to precipitate the RNA. 20µg glycogen per mL (Roche, Germany) was added as a carrier for the precipitated RNA. The sample was vortexed for 5 seconds, incubated on ice for 10 minutes then centrifuged at $12000 \times g$ for 10 minutes at 2 to 8°C. The supernatant was removed and the pellet washed with 75% v/v ethanol (VWR International, UK) in nuclease-free water (Ambion, UK), adding at least 1mL 75% ethanol per 0.75mL TRIzol Reagent. The sample was vortexed for 5 seconds and centrifuged at $12000 \times g$ for 10 minutes at 2 to 8°C. The supernatant was removed and the pellet left to partially dry then dissolved in nuclease-free water.

2.3.5.2. RNA extraction from *RNAlater* tissue samples

Tissue samples were defrosted and excess *RNAlater* (Ambion, UK) removed by dabbing the samples on blotting paper. Approximately 20mg (18 to 22mg, variation accounted for and corrected during virus quantification) of tissue was added to 700µL of Tissue Lysis Buffer (MagNA Pure LC, RNA Isolation Kit III, Roche, UK) in homogenisation tubes containing Lysing Matrix D (Q-BIOgene, UK). Tissue was homogenised by agitation in a FastPrep FP120 agitation centrifuge (Q-BIOgene, UK) for 3×45 seconds at 6500rpm, then kept at room temperature for 30 minutes to equilibrate according to the manufacturer's instructions (Ryan *et al.*, 2007). Samples were moved to -80°C for storage. Total RNA was extracted using the MagNA Pure LC, RNA extraction kit III (Roche, UK) and MagNA Pure LC robot (Roche, UK).

Genomic DNA was removed by DNase 1 (Roche, UK) treatment and purified RNA eluted with 50 μ L Roche Elution Buffer (Quan *et al.*, 2004, Ryan *et al.*, 2007).

2.3.5.3. DNA extraction, purification and concentration using phenol/chloroform /isoamyl alcohol and ethanol

DNA was extracted from aqueous solutions using phenol/chloroform/isoamyl alcohol (25:24:21, v/v) and concentration by ethanol precipitation (Moore and Dowhan, 2003). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:21, v/v, Invitrogen, UK) was added to 400 μ L of DNA solution containing no more than 1mg/mL DNA. The mix was vortexed for 5 seconds and centrifuged at 12000 \times g for 10 minutes at 4°C. The aqueous phase containing the DNA was removed, mixed with 0.5 \times volume chloroform (Sigma-Aldrich, UK), vortexed for 5 seconds and centrifuged at 12000 \times g for 10 minutes at 4°C. The aqueous phase was removed and mixed with 2.5 \times volume ice-cold 100% ethanol (VWR International, UK) and 0.1 \times volume 3M sodium acetate, pH 5.2 (Sigma-Aldrich, UK). The mix was vortexed and placed into a -20°C freezer for at least 30 minutes, followed by centrifugation at 12000 \times g for 30 minutes at 4°C. The supernatant was removed from the DNA precipitate by pipetting. The precipitate was washed once in 70% v/v ice cold ethanol in nuclease free water (Ambion, UK) at 12000 \times g for 15 minutes at 4°C. The supernatant was removed and pellet dried before resuspension in nuclease free water.

2.3.6. Reverse transcription

2.3.6.1. TaqMan Reverse Transcription Reagents

Reverse transcription using TaqMan Reverse Transcription Reagents (Applied Biosystems, UK) was carried out at a final volume of 15µL, containing 9µL TaqMan Reverse Transcription Reagent reaction mix (Appendix 1) and 6µL RNA (Quan *et al.*, 2004, Reid *et al.*, 2001, Zhang and Alexandersen, 2003). The recommended template quantity was 3ng to 0.13µg total RNA per 15µL reaction. The reactions were incubated on a thermocycler (Eppendorf, UK) at 48°C for 45 min followed by 95°C for 5 min.

2.3.7. DNA sequencing

Sequencing was performed with Dye Terminator Cycle Sequencing Quick-Start kits (Beckman Coulter, USA). Plasmid DNA templates were initially pre-heat treated at 96°C for 1 minute. A 100fmol of DNA template was added to the sequencing reaction mix containing 3.2pmol of primer, 8µL DTCS Quick Start Master Mix and nuclease free water to make up a final reaction volume of 20µL. The reaction was subjected to 30 cycles of denaturing at 96°C for 20 seconds, annealing at 50°C for 20 seconds and extension at 60°C for 4 minutes. On completion of the PCR, 5µL stop solution/glycogen mix was added to each reaction, followed by ethanol precipitation and two ethanol washes. The air dried product was resuspended in sample loading solution and analysed with an automated capillary sequencer CEQ 8800 Genetic Analysis System (Beckman Coulter, USA). Three forward and three reverse sequencing reactions were run for each DNA sample.

2.3.8. Restriction enzyme digestion of DNA

Restriction digests were performed according to the manufacturer's instructions (Promega, UK). Generally, DNA samples and plasmid DNA (200ng to 5µg) were digested in volumes of 20 to 30µL and incubated in a 37°C water bath for 2 to 15 hours.

2.3.9. Transformation of competent *E. coli*

Plasmid vectors were transformed into competent *E. coli* using a method based on the high-efficiency Hanahan transformation method (Sambrook and Russel, 2001). 50ng of plasmid DNA was added to 50µL of competent cells in a sterile tube and left on ice for 30 minutes, after which the tubes were heat shocked in a 42°C water bath for 90 seconds. The tubes were then placed back on ice for a further 2 minutes, 800µL SOC media (Appendix 1) was added and the transformation mix was incubated on a shaker at 37°C for 1 hour. Transformations were performed with undigested plasmid and digested plasmid without the insert as positive and negative controls respectively. The aliquots of cells were streaked onto Luria-Bertani agar plates (Appendix 1) containing the appropriate antibiotic and incubated for 8 to 16 hours at 37°C. Colonies were selected, suspended and incubated at 37°C in Luria-Bertani broth (Appendix 1) containing the appropriate antibiotic for 8 to 16 hours. Aliquots of bacterial cultures were diluted 1:1 (v/v) with sterile glycerol (Sigma-Aldrich, UK) and stored at -70°C.

2.3.10. Quantitative real-time reverse transcription-polymerase chain reaction

Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems, UK) as described under section 2.3.6 (Quan *et al.*, 2004, Reid *et al.*, 2003, Zhang and Alexandersen, 2004). Each 96-well reverse transcription PCR plate (Thermo Scientific, UK) contained triplicate wells of no reverse transcription controls (RT controls) and no template controls (NT controls) in addition to duplicate wells of FMDV and 28s standard RNA dilution series. RT controls consisted of known positive control RNA samples run exactly as the other quantitative rRT-PCR reactions, except that the reverse transcription enzyme was omitted. NT controls contained nuclease free water in place of RNA template. 5 μ L of cDNA was used per PCR reaction in 96-well optical reaction plates (Stratagene, UK). Duplicate wells of PCR buffer controls containing nuclease free water instead of cDNA were included on the plates. The PCR reaction was performed as described previously (Quan *et al.*, 2004, Reid *et al.*, 2001) with SA-UK-IRES-308R/SA-UK-IRES-248F primers and UK-IRES-271T probe (Appendix 2) designed by Prof. S Alexandersen specific for O UKG 34/2001 (Applied Biosystems, UK). The probe was a linear minor groove binding (MGB) TaqMan probe with fluorescent reported dye 6-carboxyfluorescein (FAM) attached to the 5' end of the probe and the quencher carboxytetramethylrhodamine (TAMRA) attached to the 3' end. The PCR reaction mix (total volume of 25 μ L/well) contained the forward and reverse primers (0.9pmol/ μ L of each), probe (0.2pmol/ μ L), and 1 \times TaqMan Universal PCR Master Mix (Applied Biosystems, UK) containing the passive reference dye 5-carboxy-X-rhodamine (ROX). The PCR was performed on a Stratagene MX3005p quantitative PCR instrument (Stratagene, USA). The thermal cycle heated the samples to 50°C

for 2 minutes for optimal uracil-N-glycosylase enzyme activity, then to 95°C for 10 minutes to activate the AMpliTaq Gold DNA polymerase. This was followed by 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C to amplify the DNA.

Stratagene MxPro software (Stratagene, USA) was used for data analysis.

Amplification plots were set to a common baseline, above which any shift in fluorescence corresponded to the change in fluorescence due to DNA amplification, using the 'adaptive method' of baseline correction with the baseline set between cycle 3 and 15. Data analysed using this method provided a more accurate estimate of the starting amount of a sample compared to a manually adjusted baseline (Oleksiewicz *et al.*, 2001, Quan *et al.*, 2004). The threshold fluorescence was set using the software algorithm amplification-based threshold method. Analysis resulted in the assignment of a threshold cycle (Ct) value to each PCR reaction which correlated with the initial target concentration. Samples with no detectable fluorescence above threshold after 50 cycles were taken to be absolutely negative (Oleksiewicz *et al.*, 2001, Quan *et al.*, 2004). Standard curves of Ct values versus known copies per standard well were generated by the software, and the quantity of copies in test wells calculated by reference to these standard curves.

2.3.11. One step real time reverse transcription-polymerase chain reaction

RNA extracted from probang samples of O1 BFS 1860 infected cattle was kindly analysed by K Ebert (IAH) using the one step FMDV diagnostic rRT-PCR. Duplicate wells containing 5µL of negative control, weak positive control, positive control and strong positive control RNA were included with sample RNA in duplicate on 96-well

optical reaction plates (Stratagene, UK). The PCR reaction was performed as described previously (King *et al.*, 2006, Reid *et al.*, 2002, Shaw *et al.*, 2007) to detect a conserved sequence within the internal ribosomal entry site using redundant primers SA-IR-219-246F/SA-IR-315-293R and SAmulti2-P-IR-292-269R TaqMan probe (Appendix 2), and a conserved sequence within the 3D region using primers Callahan 3DF/Callahan 3DR and Callahan 3DP TaqMan probe (Appendix 2). The PCR reaction mix (25µL/well) contained the forward and reverse primers (0.8pmol/µL of each), probe (0.3pmol/µL), 1 × PCR buffer (Invitrogen, UK) and 0.5µL Superscript/III Platinum Taq enzyme mix (Invitrogen, UK). The PCR was performed on a Stratagene MX3005p quantitative PCR instrument (Stratagene, USA). The thermal cycle heated the samples to 60°C for 30 minutes, then to 95°C for 10 minutes followed by 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

Stratagene MxPro software (Stratagene, USA) was used for data analysis as described under section 2.3.10 except that the threshold was manually adjusted by inspecting the amplification pots and samples were expressed as either positive or negative based on a modified cut-off Ct of 32 (Shaw *et al.*, 2007).

2.3.12. Statistical analysis of real-time PCR data quantifying FMDV genome and 28s rRNA

Statistical analysis of the data was carried out in consultation with S Gubbins, IAH, and S Abeyasekera, Statistical Services Centre, University of Reading. Minitab software (Minitab Limited, UK) was used to perform the analysis. The analysis of variance (ANOVA) general linear model (Lindman, 1974) was used to determine if

there was a statistically significant association between the FMDV genome copies expressed as FMDV copies per 10^8 copies of 28s rRNA and the amount of 28s rRNA per PCR reaction. The Fisher's exact test was used to determine if there was a statistically significant associated between the quantity of FMDV present in germinal centre samples and the type of tissue samples. The ANOVA, Tukey simultaneous test was used to compare FMDV genome copies per 10^8 copies of 28s rRNA detected in samples of six germinal centres harvested in three replicates from the different tissues examined.

2.3.13. Synthesis of FMDV O UKG 34/2001 3D sense and antisense RNA probes for *in situ* hybridization

2.3.13.1. RNA extraction and reverse transcription

Tongue vesicular epithelium from an O UKG 34/2001 infected bovine was collected at post-mortem into 50% v/v glycerol (VWR International, UK) in M25-phosphate buffer (Appendix 1). Supernatant from the homogenised epithelium was used to inoculate BTY cells (Appendix 1) kindly provided by S Wilsden (IAH). Total RNA was extracted using Trizol Reagent as described under section 2.3.5.1. Purified total RNA was reverse transcribed using Superscript III (Invitrogen, UK). An initial 10 μ L reaction containing 1 μ g/ μ L RNA, 2 μ M primer p15 (Appendix 2; MWG, UK), 100ng random hexamers (Invitrogen) and 1mM dNTP's was denatured at 68°C for 3 minutes then transferred to ice. The reaction volume was increased to 20 μ L by the addition of 1 \times Superscript III reaction buffer, 5mM MgCl₂, 10mM dithiotritol, 40 units RNase out (Invitrogen, UK) and 1 μ L Superscript III enzyme mix. The reaction was incubated at 42°C for 4 hours and terminated at 85°C for 5 minutes.

2.3.13.2. PCR amplification, digestion and ligation into pGEM-3Z vector

Primers FMDV 1F and FMDV 1R (Appendix 2) containing restriction enzyme recognition sites *EcoRI* and *BamHI* were designed to amplify the 1st 500 bases encoding the highly conserved region for the non-structural protein 3D of FMDV O UKG 34/2001. These primers were used in conjunction with the Advantage cDNA PCR Kit and Polymerase Mix (Clontech, UK). The PCR reaction mix was denatured at 94°C for 1 minute followed by 30 cycles of denaturing at 94°C for 30 seconds and annealing/extending at 68°C for 1 minute in accordance with Clontech's suggested cycling parameters. The PCR product was analysed on a 1% agarose gel (Appendix 1). After gel purification (Qiaprep Gel Extraction Kit; Qiagen, UK) and quantification (NanoDrop ND-1000 photospectrometer; Thermo Scientific, USA) the product and pGEM-3Z vector were digested with restriction enzymes *EcoRI* and *BamHI* (section 2.3.8). The digested products were analysed on a 1% agarose gel, purified, quantified and ligated using T4 DNA Ligase (Promega, UK). The vector was then transformed (section 2.3.9) into competent DH5α *E. coli* cells (kindly provided by J Seago, IAH).

2.3.13.3. Sequencing, transcription, purification and quantification

Plasmid DNA was extracted from overnight DH5α *E. coli* cell cultures (section 2.3.9) using Qiaprep Spin Miniprep Kits (Qiagen, UK). Sequencing (section 2.3.7) was performed to ensure that the insert contained the correct sequence in the correct orientation. The extracted plasmid DNA was linearised by restriction enzyme digestion (section 2.3.8). For antisense probe preparation, the plasmid DNA was

digested with restriction enzyme *EcoRI* (promega, UK) and for sense probe preparation with *BamHI* (promega, UK). To ensure high purity of linearised DNA required for the DIG RNA labelling reaction, the linear DNA product was extracted from the digestion reaction mix using phenol/chloroform/isoamyl alcohol (25:24:21, v/v) and concentrated by ethanol precipitation (section 2.3.5.3). The purified, linearised DNA was analysed on a 1% agarose gel to confirm cleavage (Appendix 1), quantified and diluted in nuclease free water. Digoxigenin–UTP (DIG-UTP) labelled RNA probes were produced by *in vitro* transcription of 1µg linearised DNA (DIG RNA Labelling Kits; Roche, UK). SP6 RNA polymerase enzyme was used for antisense probe production and T7 RNA polymerase enzyme for sense probe production. The kits included DNase I which was used to degrade the DNA template after the labelling reaction. The labelling reaction and DNA degradation were stopped with 0.2M ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, UK). Aliquots of the newly synthesised probes were stored at –80°C. Samples of each FMDV probe and the kit supplied control probe were analysed on a 1% agarose gel to quantify the output of the labelling reaction. To test the efficiency of the labelling reaction and to calculate the amount of DIG-labelled FMDV probe, serial dilutions of the FMDV probes and control labelled probe were spotted and fixed by UV-light onto Hybond-N nylon membrane (Amersham Life Science, UK). The membrane was incubated for 30 minutes at 15 to 25°C under agitation in TBST blocking buffer (Appendix 1). The membrane was removed from the blocking buffer and incubated for 30 min at 15 to 25°C in TBST blocking buffer containing alkaline phosphatase conjugated anti-digoxigenin (DIG) antibody (Roche, UK). The membrane was washed 3 times for 10 minutes under agitation in TBST blocking buffer and

transferred to detection buffer (Appendix 1) for 10 min. Substrate detection of the antibody conjugate was carried out as detailed under section 2.3.16. The optimal concentration of the probe was established by comparing the intensity of FMDV probe spots to the control probe.

2.3.14. Synthesis of bovine IgG1 sense and antisense RNA probes for *in situ* hybridization

A pCR2.1 TOPO vector (Invitrogen, UK) carrying a 686 base pair insert encoding the hinge, CH2 and CH3 domains of bovine IgG1 was kindly provided by R Aitken, University of Glasgow. The insert was removed from the vector using restriction enzymes *EcoRI* and *NotI* (section 2.3.8) and ligated into the pGEM-3Z vector using T4 DNA Ligase (Promega, UK). The vector was then transformed (section 2.3.9) into competent DH5 α *E. coli* cells (kindly provided by J Seago, IAH). Plasmid DNA was extracted from overnight DH5 α *E. coli* cell cultures (section 2.3.9) using Qiaprep Spin Miniprep Kits (Qiagen, UK). Sequencing (section 2.3.7) was performed with primers IgG1F and IgG1R (Appendix 2) to ensure that the insert contained the correct sequence in the correct orientation. DIG-UTP labelled RNA probes were prepared as described under section 2.3.13.3. *NotI* restriction enzyme digestion and T7 RNA polymerase were used for antisense RNA probe synthesis. *EcoRI* restriction enzyme digestion and SP6 RNA polymerase were used for sense RNA probe synthesis.

2.3.15. Synthesis of swine vesicular disease virus antisense RNA probes for *in situ* hybridization

A pGEM-T vector (Promega, UK) carrying cDNA from position 2414 to 3027 (region of the structural proteins 1C and 1D) of the swine vesicular disease virus (SVDV) genome was kindly provided by E Ryan, IAH (Lin *et al.*, 1997, Prato Murphy *et al.*, 1999). *Spe* I restriction enzyme digestion and T7 RNA polymerase were used for antisense RNA probe synthesis as described under section 2.3.13.3.

2.3.16. *In situ* hybridization procedure

An optimised *in situ* hybridization method was developed to detect FMDV (Prato Murphy *et al.*, 1999) and optimised for cryosections incorporating tyramide signal amplification (TSA) and alkaline phosphatase based visualisation (Yang *et al.*, 1999).

Approximately 7µm thick cryosections were prepared (Frigocut cryostat; Leica, Germany) onto Superfrost Plus microscope slides (VWR International, UK). BHK-21 cells were cultured (Appendix 1) *in vitro* directly onto slides using Chamber Slide Culture Chambers (Nunc, USA). Slides were air dried and fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich, UK) in nuclease free PBS (Ambion, UK) at 4°C for 20 minutes. Slides were rinsed with PBS for 5 minutes, dipped briefly into nuclease free water (Ambion, UK) then transferred to 100% ethanol (Sigma-Aldrich, UK) at 4°C for 5 minutes. Endogenous peroxidases were quenched by incubating the slides for 20 minutes in 1% (v/v) hydrogen peroxide (Sigma-Aldrich, UK) in methanol (Sigma-Aldrich, UK). Slides were then washed twice in PBS for 5 min.

Endogenous phosphatases were inactivated by incubation in 0.2M HCl (Sigma-Aldrich, UK) for 8 minutes. Slides were washed twice in PBS for 5 minutes then transferred to acetylation solution (Appendix 1) for 10 minutes under gentle agitation to reduce non-specific probe binding to tissue proteins (Hayashi *et al.*, 1978). Slides were washed twice with PBS for 5 min under gentle agitation and immediately covered with prewarmed pre-hybridization buffer (Appendix 1) at 60°C for at least 2 hours. Probes were mixed with hybridization buffer (Appendix 1) and incubated at 60°C for 20 minutes to ensure that the probe was evenly distributed in the buffer. The prehybridization buffer was discarded and sections covered with the hybridization buffer for incubation at 65°C for 5 min to eliminate probe secondary structure then 60°C for 14 to 16 hours.

The following post-hybridization washes were conducted under gentle agitation:

Wash solution	Temperature	Time
4×SSC and 1mM DTT	60°C	5 minutes
2×SSC and 1mM DTT	60°C	30 minutes
RNA digestion solution (Appendix 1)	37°C	30 minutes
2×SSC and 1mM DTT	60°C	30 minutes
1×SSC	60°C	30 minutes

SSC = SSC buffer (Sigma-Aldrich, UK)

DTT = Dithiothreitol (Sigma-Aldrich, UK)

For conventional chromagenic detection without TSA, the slides were washed twice for 10 minutes in TBS washing buffer (Appendix 1) then blocked in TBST blocking buffer (Appendix 1) for 30 minutes. The sections were incubated for 2 hours in a suitable dilution of sheep anti-DIG-alkaline phosphatase antibody (Roche, UK) diluted in TBST blocking buffer. Slides were washed twice for 10 minutes in TBS

washing buffer and incubated for 10 minutes in detection buffer (Appendix 1) containing 50mM MgCl₂ (Sigma-Aldrich, UK).

PerkinElmer TSA Biotin Kits (PerkinElmer, UK) were used for chromagenic detection with TSA following the post-hybridization washes. Sections were blocked for 30 minutes at room temperature with TNB buffer (Appendix 1). Sections were covered and incubated for 30 minutes with anti-digoxigenin antibody conjugated with horseradish peroxidase (Roche, UK) diluted 1:250 in TNB buffer. Slides were washed 3 times for 5 minutes in TNT buffer (Appendix 1) and incubated with biotinylated-tyramide (PerkinElmer, UK) for 5 minutes. Following three 5 minute washes in TNT buffer, the slides were incubated in the dark for 60 minutes with streptavidin conjugated with alkaline phosphatase (Roche, UK) diluted 1:750 in TNB buffer. Following incubation slides were washed 3 times for 5 minutes in TNT buffer.

The slides were incubated for 10 minutes in detection buffer (Appendix 1) followed by colour substrate solution (Appendix 1). When colour development was optimal (approximately after 2 minutes with TSA and after 30 minutes when using conventional chromagenic detection) slides were rinsed in distilled water and mounted with aqueous mounting medium (Immu-Mount; Thermo Shandon, USA).

2.3.17. Immunofluorescence confocal microscopy

All data were collected sequentially using a Leica SP2 scanning laser confocal microscope (Leica, Germany). M Windsor (IAH) kindly assisted with slide screening to detect FMDV capsid.

2.3.17.1. Immunofluorescence labelling method

Approximately 7µm thick cryosections were prepared (Frigocut cryostat; Leica, Germany) onto Superfrost Plus microscope slides (VWR International, UK).

Sections were air dried and fixed in 100% acetone (Sigma-Aldrich, UK) at –20°C for 5 minutes. Slides were air dried for 20 minutes and used immediately.

Cell cultures were prepared for microscopy onto 13mm cover glass (VWR international, UK) and fixed for 45 minutes in 4% (w/v) paraformaldehyde (Sigma-Aldrich, UK) in PBS (CSU, IAH). Cells were made permeable for internal staining by incubation with 0.1% (v/v) Triton X-100 (Sigma-Aldrich, UK) in PBS for 15 minutes under agitation followed by three 15 minute washes in PBS.

Non specific binding of detection antibodies was blocked by incubation with 5% (v/v) normal goat serum (Sigma-Aldrich, UK) in Ca/Mg free PBS (CSU, IAH) for 20 minutes. Sections were blotted dry and incubated with the primary antibody for 30 minutes at room temperature. Primary and secondary antibodies were diluted in 5% normal goat serum in Ca/Mg free PBS. For purified mouse anti-bovine antibodies a solution of 1 to 10µg/mL was initially used. For tissue culture supernatants a starting dilution of 1:10 was initially used. Slides were washed 5 times in Ca/Mg free PBS

and incubated with the secondary goat anti-mouse isotype-specific secondary antibody (Alexa fluor; Molecular Probes, UK) at a working dilution of 1:500 for 20 minutes in the dark. Slides were washed as before and incubated for 15 minutes with a 1:20000 dilution of the DNA-binding stain 4'-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, UK) in Ca/Mg free PBS. Slides were washed in Ca/Mg free PBS and mounted. Vectorshield (Vector Laboratories, UK) was used to mount slides prepared with Alexa fluor 568. Prolong gold (Invitrogen, UK) or Fluoromount G (SouthernBiotech, UK) was used for all other secondary antibodies. For each tissue section labelled with antibodies of interest, additional sections of the same tissue were labelled with isotype matched control antibodies, with secondary anti-mouse fluorochrome conjugated antibody only and without primary antibody as controls. Tissue sections from infected animals were also labelled in parallel with sections from non-infected control animals.

2.3.17.2. List of primary antibodies

Table 1. Primary antibodies.

Antibody	Specificity	Isotype	Reference
AD10	FMDV capsid	IgG1	(Juleff <i>et al.</i> , 2008)
AV29	Isotype control (chicken antigen)	IgG2b	Unpublished ¹
AV48	Isotype control (chicken antigen)	IgM	Unpublished ¹
BF8	FMDV capsid	IgG2b	(Juleff <i>et al.</i> , 2008)
CC21	CD21	IgG1	(Howard and Morrison, 1991)
CC51	CD21	IgG2b	(Howard and Morrison, 1991)
CC158	MHC class II	IgG2a	(Howard and Morrison, 1991)
CCG33	CD14	IgG1	(Sopp <i>et al.</i> , 1996)
CCG36	CD32	IgG1	Unpublished ²
CCG37	CD32	IgG2a	Unpublished ²
CNA.42	Light zone FDCs	IgM	(Lefevre <i>et al.</i> , 2007) ³
D46	Fibrinogen	IgG2a	(Lefevre <i>et al.</i> , 2007) ⁴
D9	FMDV VP1 (1D)	IgG2a	(Brocchi <i>et al.</i> , 1983) ⁵
FC6	FMDV capsid	IgG1	(Juleff <i>et al.</i> , 2008)
IB11	FMDV capsid	IgG1	(Juleff <i>et al.</i> , 2008)
ILA21	MHC class II	IgG2a	(Schuberth <i>et al.</i> , 1996) ⁶
ILA156	CD40	IgG1	(Haas <i>et al.</i> , 2001) ⁶
TRT1	Isotype control (turkey rhinotracheitis virus)	IgG1	(Cook <i>et al.</i> , 1993)
TRT3	Isotype control (turkey rhinotracheitis virus)	IgG2a	(Cook <i>et al.</i> , 1993)
TRT6	Isotype control (turkey rhinotracheitis virus)	IgG2b	(Cook <i>et al.</i> , 1993)
2C2	FMDV 3A	IgG2a	(De Diego <i>et al.</i> , 1997) ⁵
3C1	FMDV 3C	IgG2a	(Brocchi <i>et al.</i> , 1998) ⁵
10D5	$\alpha\text{v}\beta 6$	IgG2a	(Monaghan <i>et al.</i> , 2005) ⁷

¹ AV29 and AV48 are MAbs directed against chicken antigens provided by F Davison and produced at the IAH (Russell *et al.*, 1997).

² CCG36 and CCG37 MAbs were kindly provided by C Howard and produced at the IAH.

³ CNA.42 was kindly provided by G Delsol, Toulouse, CHU Purpan, Laboratoire d'anatomie et cytologie pathologiques, France.

⁴ D46 was kindly provided by E Lefevre and produced at the IAH.

⁵ D9, 2C2 and 3C1 were kindly provided by E Brocchi, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna Reparto Biotecnologie, Italy.

⁶ ILA21 and ILA156 were kindly provided by the International Livestock Research Institute, Kenya.

⁷ MAb 10D5 was procured from Chemicon, UK.
All other MAbs were produced at the IAH.

2.3.17.3. Monoclonal antibodies specific for conformational, non-neutralising epitopes of the FMDV capsid

B Jones (IAH) kindly provided a panel of culture fluid from antibody-secreting hybridoma cells derived from mice immunised with 146 S FMDV type O1 antigen (Sucrose gradient purified FMDV was kindly provided by N Ferris, IAH). The panel was screened by M Windsor and L Reid (IAH) using a sandwich ELISA with plates coated with O1 Manisa antigen. Selected MAbs were screened by immunofluorescence confocal microscopy (section 2.3.17) on vesicular lesion cryosections harvested from FMDV O UKG 34/2001 infected cattle and in parallel on non-infected control tissue cryosections. The selected MAbs were also screened on BHK-21 cells (Appendix 1) fixed 5 hours after FMDV O UKG 34/2001 infection at multiplicity of infection (MOI) 10, and on mock-infected cells (PBS) by immunofluorescence confocal microscopy. The cryosections and cells were screened in combination with MAb 2C2 and 3C1 (section 2.3.17.2) as positive controls.

Mouse MAbs IB11, FC6, AD10 and BF8 were selected and screened by virus neutralising antibody test performed by P Hamblin (IAH) as described in the Office International des Epizooties (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 5th edition, 2004. Immunoprecipitation analysis was performed by M Windsor, IAH, as previously described (Rouiller *et al.*, 1998). BHK-21 cells (Appendix 1) were infected with O1BFS at MOI 5 for four hours in total and pulsed with 35S methionine/cysteine for two of these hours. Cells were lysed and immunoprecipitated with D9, IB11, FC6, AD10, BF8 and TRT1 (section 2.3.17.2)

coupled to protein G sepharose. The MAbs were subsequently screened by western blotting analysis by M Windsor (IAH).

2.3.17.4. Detecting FMDV immune complexes

The ability of MAb IB11 to detect FMDV immune complexes was evaluated *in vitro*. Serum was collected from an animal previously infected with O UKG 34/2001 and from a naïve animal. The serum samples were heat treated at 56°C for 35 minutes and diluted 1/100 in a serum free solution of FMDV type O at 2.2×10^7 pfu/mL at room temperature for 30 minutes to form immune complexes (Robinson, 2008). Approximately 1×10^5 mouse fibroblast 3T3 cells (Appendix 1) expressing bovine CD32 (section 2.3.18) were fixed onto glass cover slips in 1% (w/v) paraformaldehyde (Sigma-Aldrich, UK) in PBS (CSU, IAH) for 15 minutes. The cells were washed three times in PBS for 15 minutes under agitation. The cells were incubated at room temperature in serum free media containing a 1/16 dilution of the virus-serum solutions for 30 minutes under agitation. The cells were washed three times in PBS for 15 minutes under agitation, fixed in 4% (w/v) paraformaldehyde in PBS for 35 minutes and labelled for confocal microscopy (section 2.3.17).

2.3.18. Mouse fibroblast 3T3 cells expressing bovine CD32

2.3.18.1. PCR amplification and TA cloning into pcDNA3.1/V5-His-TOPO vector

A bacterial colony containing cDNA clone IMAGE: 8083027 of *Bos taurus* low affinity IgG Fc receptor (CD32/FcγRII) mRNA was procured from Geneservice limited, UK (NCBI accession BC113215). The colony was streaked onto Luria-Bertani agar plates and colonies were selected for overnight culture in Luria-Bertani

broth as described under section 2.3.9. Plasmid DNA was extracted from overnight cell cultures using Qiaprep Spin Miniprep Kits (Qiagen, UK). Amplification of DNA was performed using *Pfu* DNA polymerase (Stratagene, UK). Each 100µL reaction mix contained 200ng of DNA template (NanoDrop ND-1000 photospectrometer, Thermo Scientific, USA) and 0.5µM forward and reverse primers CD321F and CD321R (Appendix 2). The samples were denatured at 94°C for 45 seconds, annealed at 55°C for 45 seconds and extended at 72°C for 1 min during 30 cycles in accordance with Stratagene's suggested cycling parameters. The 3' A-overhangs were added post-amplification by incubating 50µL of the PCR reaction with 1 unit *Taq* polymerase (Invitrogen, UK) at 72°C for 10 minutes. After gel purification (Qiaprep Gel Extraction Kit; Qiagen, UK) the product was cloned into the pcDNA3.1/V5-His-TOPO mammalian expression vector (Invitrogen, UK) by TA cloning performed at a final salt concentration of 200mM NaCl and 10mM MgCl₂. The vector was transformed (section 2.3.9) into competent One Shot TOP10 *E. coli* (Invitrogen, UK).

2.3.18.2. Digestion, ligation into pcDNA6/V5-His-ABC vector and sequencing

Plasmid DNA was extracted from overnight *E. coli* cell cultures using Qiaprep Spin Miniprep Kits (Qiagen, UK). The extracted plasmid and pcDNA6/V5-His-ABC vector were digested (section 2.3.8) with restriction enzymes *Hind*III and *Not*I (Promega, UK). The digested products were analysed on a 1% agarose gel (Appendix 1), gel purified (Qiaprep Gel Extraction Kit, Qiagen, UK) and ligated using T4 Ligase (Promega, UK). The vector was then transformed (section 2.3.9) into competent One Shot TOP10 *E. coli* (Invitrogen, UK). Sequencing (section 2.3.7)

was performed to ensure that the inserts contained the correct sequence in the correct orientation.

2.3.18.3. Transfection of mouse fibroblast 3T3 cells and selection of mouse fibroblast 3T3 cells expressing bovine CD32

Plasmid DNA was transfected into mouse fibroblast 3T3 cells (Appendix 1) using Lipofectamine 2000 (Invitrogen, UK). Stable cell lines were selected with G418 (1mg/mL, Gibco, UK) or Blasticidin S HCl (20µg/mL, Invitrogen, UK) approximately 24 hours after transfection. The degree of CD32 expression was evaluated by fluorescence activated cell sorting (FACS) analysis (section 2.3.20) and by immunofluorescence confocal microscopy (section 2.3.17) using primary antibodies specific for bovine CD32 (Table 1).

2.3.19. BHK-21 cells expressing CD32 and CD32tail- mutant

2.3.19.1. Mutagenesis

The *Bos taurus* low affinity IgG Fc receptor (CD32/FcγRII) amino acid sequence (NCBI accession BC113215) was aligned with the *Homo sapien* amino acid sequence (Stuart *et al.*, 1987) to identify the extracellular, transmembrane and cytoplasmic domains of bovine CD32. Point mutations were chosen at the 5' end of the cytoplasmic domain to introduce two stop codons to replace an arginine and a lysine code. These point mutations were based on a *Homo sapien* CD32 mutant lacking the cytoplasmic domain (Peltz *et al.*, 1988, Tuijnman *et al.*, 1992). The QuickChange Site-Directed Mutagenesis Kit performed with *Pfu* Turbo DNA polymerase (Stratagene, UK) was used to introduce point mutations with

CD32Fmutant and CD32Rmutant primers (Appendix 2). Both pcDNA3.1/V5-His-TOPO and pcDNA6/V5-His-ABC containing the CD32 insert were mutated.

Following temperature cycling, the products were treated with *DpnI* endonuclease specific for methylated DNA for parental DNA template digestion. The remaining vectors containing the desired mutations were then transformed (section 2.3.9) into XL1-Blue (Invitrogen, UK) cells. Sequencing (section 2.3.7) was performed to ensure that the inserts contained the correct sequence in the correct orientation.

2.3.19.2. Transfection of BHK-21 cells and selection of BHK-21 cells expressing bovine CD32

BHK-21 cells (Appendix 1) were transfected and selected as described under section 2.3.18.3. In addition, the ability of BHK-21 cells or BHK-21 cells expressing either CD32 or the CD32tail– mutant, to mediate efficient endocytosis of immune complexed ovalbumin was compared (Miettinen *et al.*, 1992). IgG was purified from heat treated sera (56°C for 35 minutes) of ovalbumin vaccinated cattle using a HiTrap protein G HP column (Amersham Biosciences, UK). Fluorescein isothiocyanate (FITC) ovalbumin (Molecular Probes, UK) was suspended in PBS (CSU, IAH) to a final concentration of 25mg/mL. Purified antibody (4mg/mL) was diluted 1/50 in the resuspended ovalbumin and incubated at room temperature for 30 minutes to form immune complexes (Robinson, 2008). 5×10^5 cells were held on ice for 15 minutes then exposed to FITC-ovalbumin, or FITC-ovalbumin immune complexes at 4°C for 1 hour. Cells were subsequently held on ice to assess background fluorescence, or at 37°C to measure uptake. After 30 minutes cells were

washed extensively with ice cold FACS wash buffer before immediate flow cytometric analysis (section 2.3.20) using ice cold solutions.

2.3.19.3. Virus neutralising antibody test

Serum samples from 13 days or more post FMDV O UKG 34/2001 infection were heat inactivated at 56°C for 1 hour and analysed by the virus neutralising antibody test to measure the ability of the serum to neutralise a fixed dose of virus on BHK-21 cells (Appendix 1) and BHK-21 cells expressing CD32. The tests were performed as described in the Office International des Epizooties (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 5th edition, 2004 (Golding *et al.*, 1976), under the guidance of P Hamblin, IAH, with modifications. The tests were performed in triplicate wells of flat-bottomed Nunc TC microwell 96 FSI plates (Fisher Scientific, UK). The test sera was diluted across the plate in serum free medium, 50µL of titrated O UKG virus stock (P Hamblin, IAH) was added to each well and plates were incubated at 37°C for 1 hour. The virus stock was titrated on BTY cells (Snowdon, 1966) and diluted so that each 50 µL unit volume of virus suspension contained 100 TCID₅₀. A cell suspension at 1×10^6 cells/mL was made up in medium containing 10% (v/v) fetal calf serum (Autogen Bioclear, UK). 50µL of the cell suspension (0.5×10^5 cells) was added to each well. The following duplicate control wells were included on the plate to ensure the assays were valid: negative serum (kindly provided by P Hamblin, IAH), serum free medium and cells, medium and cells. The plates were incubated at 37°C with readings taken at 24, 48 and 72 hours for cytopathic effect. After 72 hours the plates were stained with 0.4% (w/v)

naphthalene black (Searle Diagnostics, UK) in PBS (CSU, IAH) containing 8% (w/v) citric acid crystals (Sigma-Aldrich, UK).

2.3.20. Flow cytometry

2.3.20.1. Flow cytometry to detect surface proteins

Adherent cells were detached with non-enzymatic Cell Dissociation Solution (Sigma-Aldrich, UK) to minimise damage to surface proteins. Cell suspensions were stained with MAbs as described previously (Howard *et al.*, 1988, Howard *et al.*, 1989). Approximately 3×10^5 cells per well (U bottom 96 microwell plates; Sigma-Aldrich, UK) were stained for flow cytometric analysis. All washes and antibody dilutions were carried out in FACS wash buffer (Appendix 1). Cells were pelleted and washed once by centrifugation at $250\times g$ at 8°C for 4 minutes, before staining with the appropriate primary antibodies in conjunction with isotype control primary antibodies, for 15 minutes at room temperature (Table 1). Unbound primary antibody was removed by washing the cells twice before incubation with goat anti-mouse isotype-specific secondary antibody (Alexa fluor; Molecular Probes, UK) for 15 minutes at room temperature in the dark. Following two further washes the cells were fixed in 1% (w/v) paraformaldehyde (Sigma-Aldrich, UK) in PBS (CSU, IAH) at room temperature. Fluorescence data were collected using a Becton Dickinson FACScalibur with Cellquest software (Becton Dickinson, UK). Cells were gated on their FSC/SSC profile with a minimum of 10000 viable cells being collected in each sample and results were analysed using FCS Express version 3 (De Novo Software, US).

2.3.20.2. Flow cytometry to detect intracellular proteins

To detect intracellular proteins, cells were transferred to 96-well plates before fixation in 1% (w/v) paraformaldehyde in PBS for 15 minutes. The cells were then permeabilised by washing twice in FACS wash buffer containing 0.1% saponin (Sigma-Aldrich, UK). Staining proceeded as per detection of surface proteins with the exception that all washes were carried out in the presence of 0.1% saponin, and all antibodies were diluted in FACS wash buffer containing 0.1% saponin.

2.3.21. Virus isolation procedures

2.3.21.1. Tissue homogenisation

Tissue samples were homogenised manually by grinding in sterile sand with a mortar and pestle in a 10% (w/v) suspension of M25-phosphate buffer (Appendix 1). The suspension was either centrifuged at 1800×g for 10 minutes or treated with 50% (v/v) Freon (Sigma-Aldrich, UK) (Alexandersen *et al.*, 2002) or n-octyl- β -d-glucopyranoside (NOG, Sigma-Aldrich, UK) before centrifugation. The tissue supernatants were removed for further processing.

NOG was added to the tissue homogenate to solubilise membrane proteins. NOG was added to a final concentration of 30mM and incubated on ice for 20 minutes (Han and Tanzer, 1979, Lazo and Quinn, 1980). Following centrifugation, the supernatant was passed through a 0.45 μ m filter and dialysed using a 30000 molecular weight cut off Slide-A-Lyzer Dialysis Cassette (Thermo Scientific, USA) in M25-phosphate buffer at 4°C overnight (Saito and Tsuchiya, 1984). The dialysed

solution was removed for further processing or concentrated using a 30000 molecular weight cut off Vivaspin Column (Sartorius, UK) before further processing.

2.3.21.2. Low density cell preparations

Tonsil and lymph node samples were placed in petri dishes, cut into small blocks and teased apart using forceps, needles and steel mesh. A portion of the tissue cell preparations were digested with RPMI (Roswell Park Memorial Institute, CSU, IAH) containing 10% (v/v) fetal calf serum (Autogen Bioclear, UK), 4mM Glutamine, 10U/mL penicillin, 10U/mL streptomycin (CSU, IAH), 5mM EDTA (pH 7.4, Sigma-Aldrich, UK), 0.1mg/mL DNase type 1 (Sigma-Aldrich, UK) and 2mg/mL collagenase type 4 (Sigma-Aldrich, UK). Digestion was performed at 4°C under agitation for 1 hour (Schriever *et al.*, 1989). Digested and non-digested cell preparations were centrifuged at 650×g for 25 minutes at 8°C over a discontinuous gradient of 1.02g/mL and 1.04g/mL Percoll (Sigma-Aldrich, UK). Cells were collected from the interphase and washed twice in PBS (CSU, IAH) at 300×g for 8 minutes at 8°C.

2.3.21.3. Virus isolation on CD32 expressing cells

Bovine monocyte-derived macrophages (MΦ) were generated from CD14⁺ PBMC following a protocol developed by L Robinson, IAH, using bovine recombinant granulocyte-macrophage colony-stimulating factor (Norimatsu *et al.*, 2003). Isolated PBMC (section 2.3.3.1) were mixed with anti-human CD14 microbeads (Miltenyi Biotech, UK) at 25µL per 10⁸ cells and incubated at room temperature for 10 minutes. The cells were then washed twice in PBS (CSU, IAH) by centrifugation at

250×g at 8°C for 8 minutes and resuspended in 3mL of chilled column wash buffer [FACS sheath fluid (BD Biosciences, UK) with 2% v/v fetal calf serum (Autogen Bioclear, UK), 0.22µm filtered]. The MidiMACS LS column (Miltenyi Biotech, UK) was placed in a magnet and washed with 3mL of column wash buffer to remove preservatives before the labelled cells were added. Trapped, unlabelled cells were flushed through with a total of 7.5mL column wash buffer. To collect the bound, labelled cells the column was removed from the magnet and 5mL chilled MΦ medium [RPMI-1640 (Gibco, UK), 10% v/v fetal calf serum, 50µg/mL gentamycin (Sigma-Aldrich, UK), 0.5µM 2-mercaptoethanol (Sigma-Aldrich, UK), 0.2U/mL bovine recombinant granulocyte-macrophage colony-stimulating factor (Serotec, UK)] was pushed through. Cells were counted on a haemocytometer (Assistant, Germany) and their viability assessed by trypan blue staining (Sigma-Aldrich, UK). Freshly isolated monocytes were seeded into culture vessels at 1×10^6 cells per mL MΦ medium and incubated at 37°C, 5% CO₂. After 3 days fresh medium was added to cells. Cells were harvested at 6 days with Cell Dissociation Solution (Sigma-Aldrich, UK).

MΦ, and BHK-21 cells (Appendix 1) expressing CD32 (section 2.3.19) were prepared in 24 well plates on glass cover slips and as monolayers in six well plates. To assess the suitability of CD32 expressing cells for detecting lymphoid tissue associated FMDV, cell monolayers in 6 well plates were spiked with 100µL homogenised mandibular lymph node or palatine tonsil supernatants from a control animal before incubation with dilutions of FMDV, immune complexed FMDV, or

mock-infected (section 2.3.17.4). Cells were exposed for 6 hours before flow cytometry to detect FMDV 3A (section 2.3.20.2, Table 1).

CD32 expressing cells were inoculated with tissue homogenates and cell suspensions from infected animals, prepared as described under sections 2.3.21.1 and 2.3.21.2. After 6 hours at 37°C the glass cover slips were labelled for immunofluorescence confocal microscopy (section 2.3.17) to detect FMDV 3A. The cell cultures in 6 well plates were either used for flow cytometry (section 2.3.20) or scraped and suspended in culture fluid for virus isolation using bovine thyroid (BTY) cells (section 2.3.21.4).

2.3.21.4. Virus isolation on bovine thyroid cells

The infectivity of probang samples, tissue homogenates and cell suspensions prepared as described under sections 2.3.21.1 and 2.3.21.2 and CD32 expressing cell suspensions (section 2.3.21.3) was determined by inoculation of monolayers of primary BTY cells (Appendix 1) (Snowdon, 1966). Two hundred µL of the supernatant or suspension was added to each monolayer tube of BTY cells kindly provided by S Wilsden (IAH). Three tubes were used per sample and incubated at 37°C on roller drums. Cell monolayers were examined for cytopathic effect at 24, 48 and 72 hours post inoculation. If there was no cytopathic effect after 72 hours, the cell culture supernatant was used to inoculate a second batch of BTY tubes. An ELISA, kindly performed by G Hutchings (IAH) was used to confirm the presence of FMDV (Ferris and Dawson, 1988).

2.4. Results

2.4.1. Histology

The morphological characteristics of the lymphoid tissue associated with the soft palate, palatine tonsils and pharyngeal tonsils, and the germinal centre morphology of the spleen, mandibular, lateral retropharyngeal and bronchial lymph nodes were examined on H&E stained and immunofluorescence labelled sections harvested 15 days post-contact infection and from non-infected control animals (IAH, Compton).

The soft palate forms part of the roof of the mouth directly behind the hard palate, between the oral cavity and pharynx (Liebler-Tenorio and Pabst, 2006). The pharyngeal surface of the soft palate (referred to as the dorsal soft palate) is covered with respiratory epithelium (there is a transition from rostral to caudal of pseudostratified columnar epithelium to stratified, squamous, non-keratinised epithelium) which is continuous with that of the nasopharynx. The organised mucosa-associated lymphoid tissue (MALT) of the dorsal soft palate harvested from FMDV infected animals contained distinct secondary follicles characterised by germinal centres, the germinal centres were orientated with the light zone towards the apical surface (Figure 4). The organised MALT was sparsely distributed in the dorsal soft palate harvested from control animals, however, the morphology of the MALT was as described above for the infected animals.

The oral surface of the soft palate (referred to as the ventral soft palate) is covered with stratified, squamous, keratinised epithelium continuous with the epithelium of the oral cavity. The tonsils of the soft palate consist of cryptolymphatic units that are

associated with the ventral soft palate. The cryptolymphatic units consist of epithelial crypts (invaginations of stratified, squamous, non-keratinised epithelium forming blind ended crypts) surrounded by lymphoid follicles and interfollicular areas. Germinal centres were observed in the cryptolymphatic units harvested from FMDV infected animals and from non-infected control animals (Figure 4).

The palatine tonsils are located within the lamina propria of the lateral oropharyngeal walls. The stratified, squamous, non-keratinised epithelium forming the pharyngeal wall, invaginates into the tonsil to form the tonsillar sinus and blind-ended crypts (Palmer *et al.*, 2009). The sub-epithelial compartments of the palatine tonsils harvested from FMDV infected animals contained germinal centres, the germinal centres were orientated with the light zone towards the epithelial crypts (Figure 5). Palatine tonsils harvested from non-infected control animals contained fewer germinal centres than those harvested from FMDV infected animals, however, the morphology of the palatine tonsil was as described above for the infected animals.

The pharyngeal tonsils are located in the roof of the nasopharynx and are covered by pseudostratified columnar epithelium. Pharyngeal tonsils harvested from FMDV infected animals contained germinal centres in the absence of crypts. The germinal centres were orientated with the light zone towards the epithelium (Figure 5). Pharyngeal tonsils harvested from non-infected control animals contained fewer germinal centres than those harvested from FMDV infected animals, however, the morphology of the pharyngeal tonsil was as described above for the infected animals.

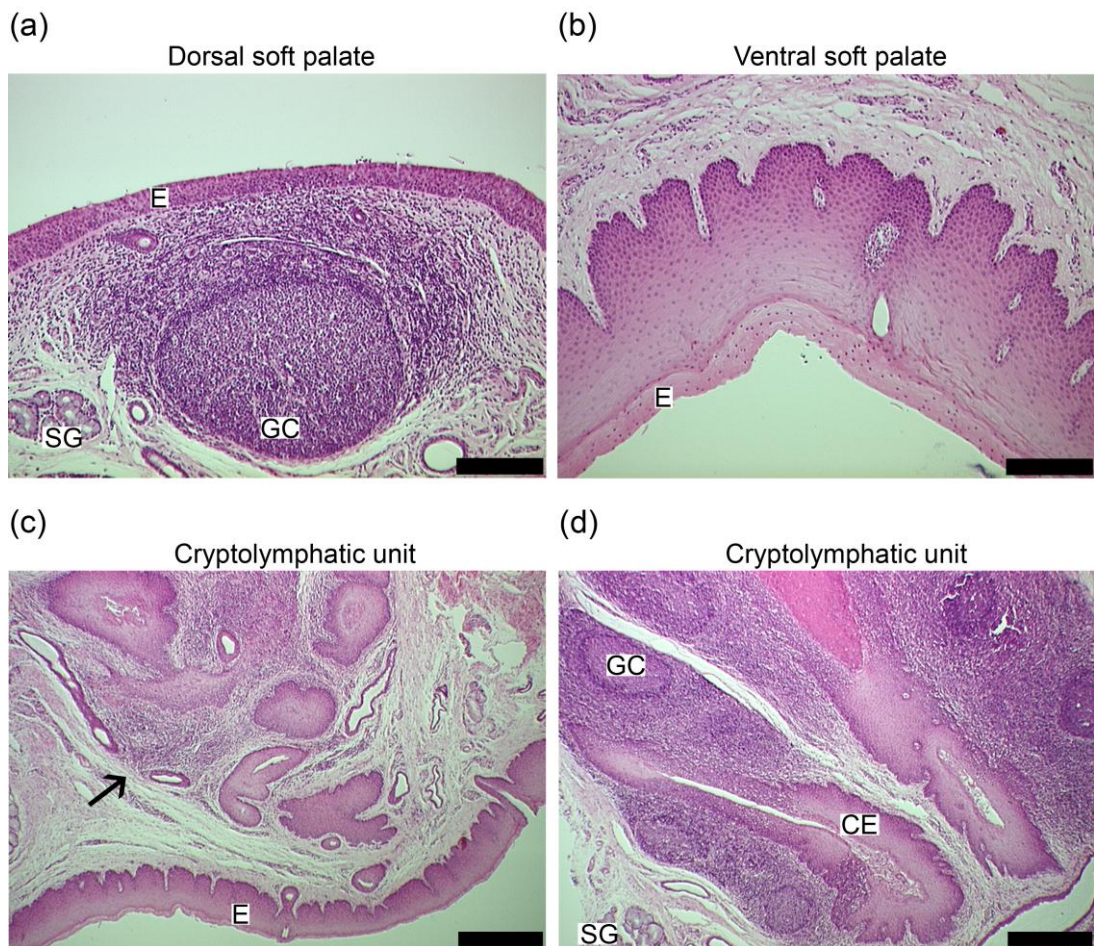


Figure 4. H&E stained sections of soft palate.

H&E stained sections of soft palate harvested 15 days post-intradermolingual challenge. (a) Section of the dorsal soft palate. Salivary glands (SG) and germinal centres (GC) were located within the connective tissue of the lamina propria below the respiratory epithelium (E). The germinal centres were orientated with the light zone towards the apical surface. (b) Section of the ventral soft palate highlighting the stratified, squamous, keratinised epithelium (E). (c) Cryptolymphatic unit (black arrow) located in the lamina propria below the epithelium of the ventral soft palate (E). (d) Germinal centres (GC) were associated with the crypt epithelium (CE) within the cryptolymphatic units. Salivary glands (SG) were located within the connective tissue of the lamina propria surrounding the cryptolymphatic units. Scale bars represent: (a) and (b), 200 μ m; (c) and (d), 500 μ m.

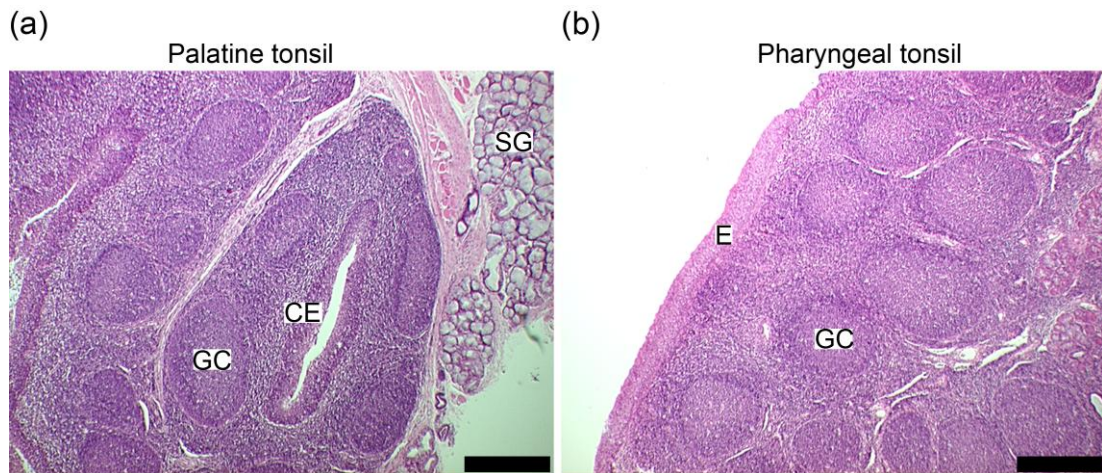


Figure 5. H&E stained sections of palatine and pharyngeal tonsils.

H&E stained sections of palatine tonsil and pharyngeal tonsil harvested 15 days post-intradermolingual challenge. (a) The germinal centres (GC) of the palatine tonsils were orientated with the light zone towards the stratified, squamous, non-keratinised crypt epithelium (CE). Salivary glands (SG) were located in the connective tissue within the lamina propria of the pharyngeal wall. (b) The germinal centres (GC) of the pharyngeal tonsil were orientated with the light zone towards the pseudostratified columnar epithelium (E). Scale bars represent 500µm.

The morphology of the mandibular and lateral retropharyngeal lymph nodes harvested from FMDV infected cattle was typical of enlarged inflammatory lymph nodes consistent with a reactive process (Willard-Mack, 2006), in comparison to the nodes harvested from non infected control animals, with follicular hyperplasia and prominent germinal centres within secondary follicles (Figure 6). Mandibular and lateral retropharyngeal lymph nodes harvested from non-infected control animals contained fewer germinal centres than those harvested from FMDV infected animals, however, the morphology of the lymph node was as described above for the infected animals.

The bronchial lymph nodes harvested from FMDV infected cattle were only mildly reactive compared to that of control animals, containing a small number of prominent germinal centres compared to the mandibular and lateral retropharyngeal lymph nodes of infected animals (Figure 6).

The spleens of FMDV infected cattle were only mildly hyperplastic and the morphology was similar to that of control animals, with a small number of prominent germinal centres associated with the splenic white pulp (Figure 7).

The microanatomy of germinal centres within harvested lymphoid tissues was examined by immunofluorescence confocal microscopy. The microanatomy of the germinal centres was similar in all the lymphoid tissue harvested during the study with clearly distinguishable dark and light zones (Figure 8), with the light zone characteristically associated with a greater degree of CD21 expression (Imal and

Yamakawa, 1996). The integrin $\alpha\text{v}\beta 6$ was not detected within germinal centres (Figure 9). Interestingly, integrin $\alpha\text{v}\beta 6$ expression was detected on cells in the tonsillar crypts (Figure 9).

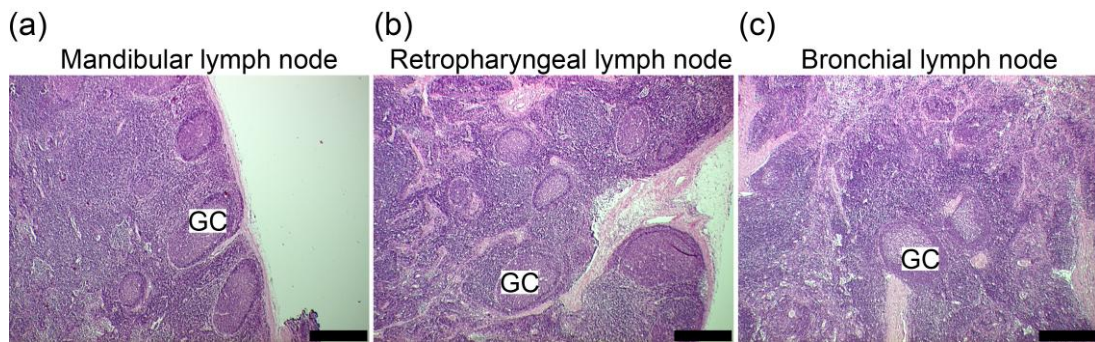


Figure 6. H&E stained sections of mandibular, lateral retropharyngeal and bronchial lymph nodes.

H&E stained sections of mandibular, lateral retropharyngeal and bronchial lymph nodes harvested 15 days post-intradermolingual challenge. (a) Mandibular lymph node and (b) lateral retropharyngeal lymph node sections with prominent germinal centres (GC) associated with secondary follicles. (c) The bronchial lymph nodes harvested from FMDV infected cattle contained a small number of prominent germinal centres (GC) compared to the mandibular and lateral retropharyngeal lymph nodes. Scale bars represent 500 μ m.

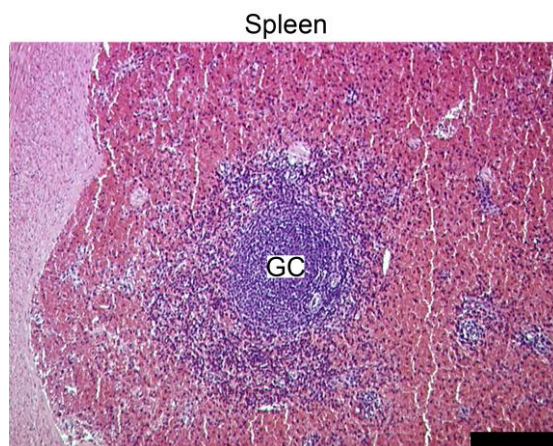


Figure 7. H&E stained spleen section.

H&E stained spleen section harvested 15 days post-intradermolingual challenge highlighting a germinal centre (GC) associated with the splenic white pulp. Scale bar represents 200 μ m.

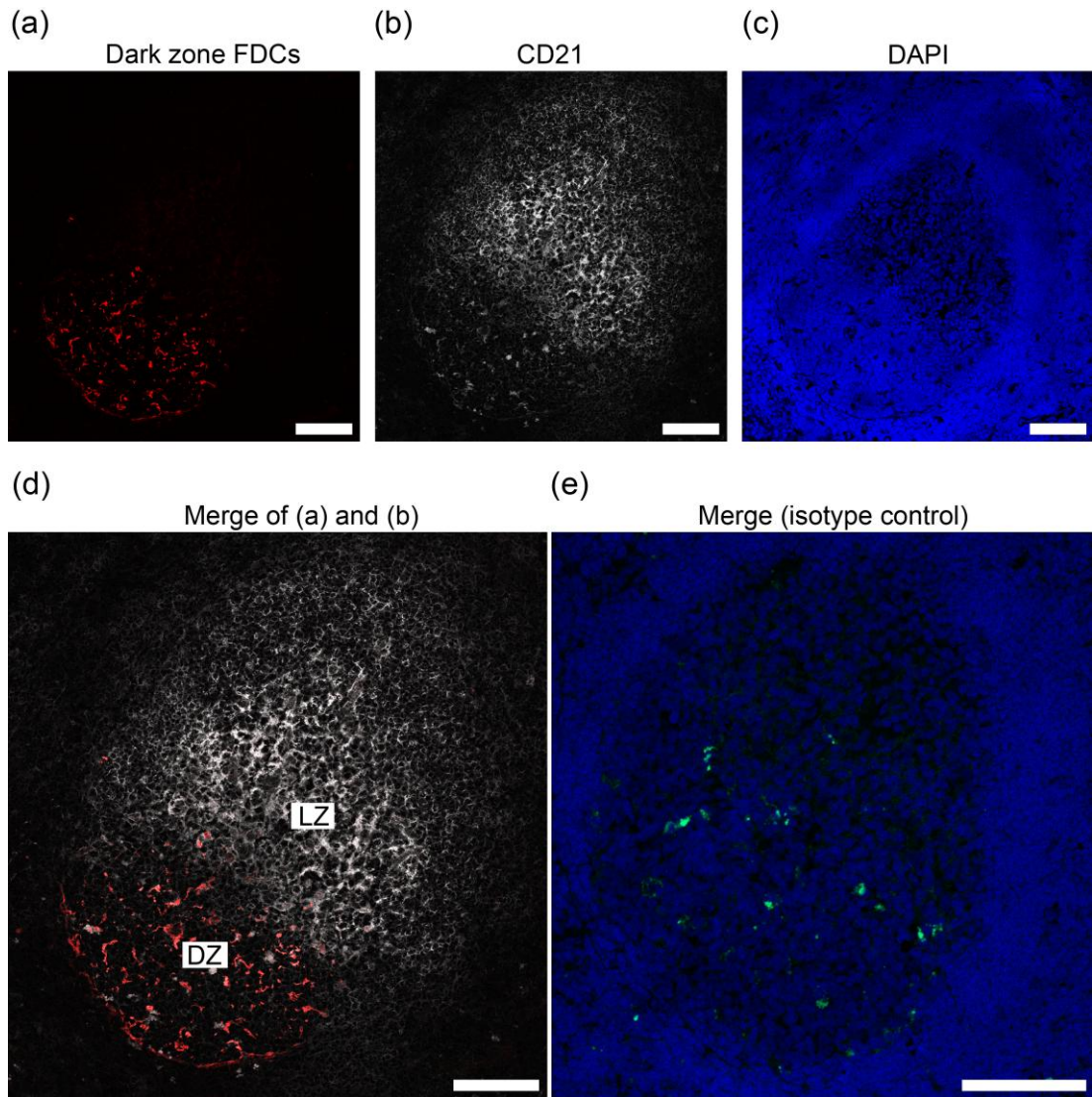


Figure 8. Germinal centre microanatomy.

Mandibular lymph node cryosections harvested from an animal 38 days post-contact infection. (a) Dark zone FDCs stained red (anti-fibrinogen MAb D46). (b) CD21 expressing cells stained gray (anti-CD21 MAb CC51). (c) Nuclei stained blue (DAPI). (d) Merge image of (a) and (b). The dark zone (DZ) is stained red. The light zone (LZ) is characterised by a high degree of CD21 expressing cells (gray). (e) Merge image of a cryosection stained with isotype control MAbs (anti-turkey rhinotracheitis virus MAbs TRT3 and TRT6) highlighting the high degree of autofluorescence associated with bovine germinal centres. Nuclei stained blue (DAPI). Scale bars represent 100µm.

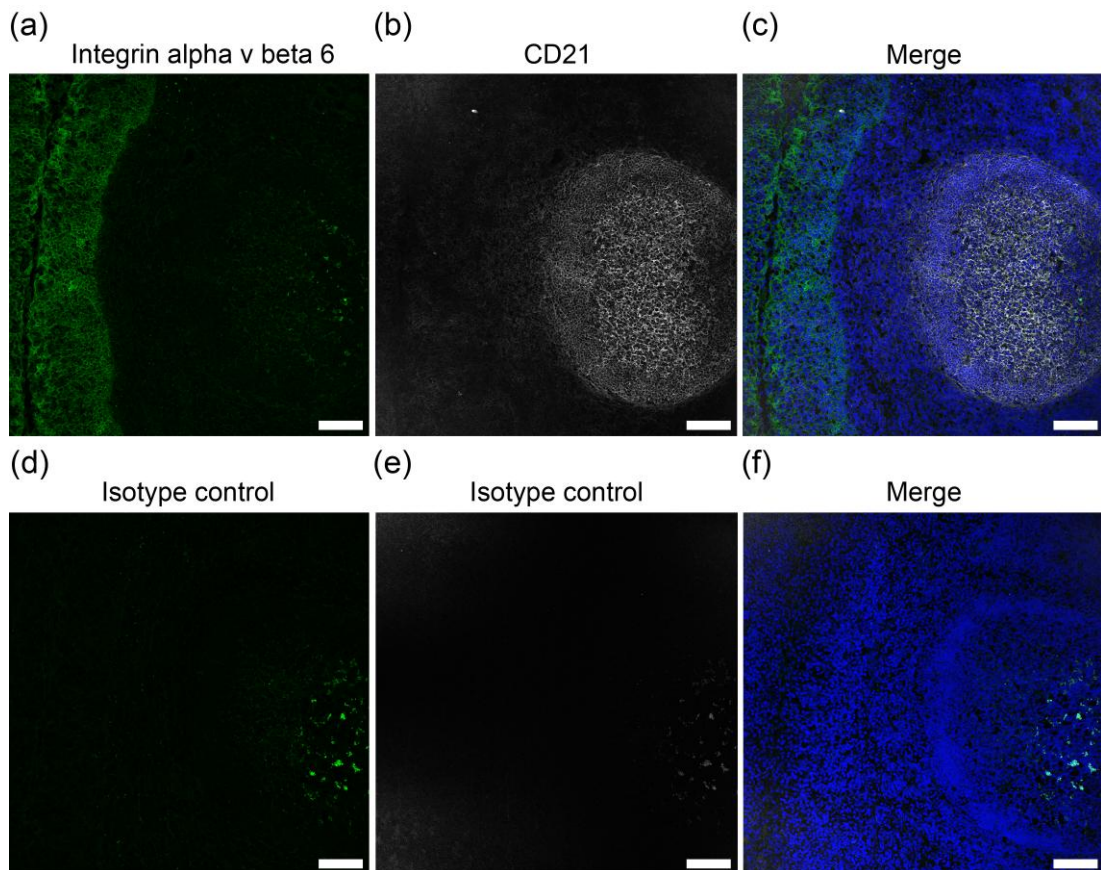


Figure 9. Integrin $\alpha\text{v}\beta 6$ expression in the palatine tonsil.

Palatine tonsil cryosections harvested from an animal 38 days post-contact infection. (a) Palatine tonsil crypt epithelium cells express the $\alpha\text{v}\beta 6$ integrin (green, anti- $\alpha\text{v}\beta 6$ MAB 10D5). Green fluorescence in the adjacent germinal centre is due to autofluorescence associated with bovine germinal centres. No $\alpha\text{v}\beta 6$ was detected in germinal centres. (b) CD21 expressing cells stained gray (anti-CD21 MAb CC51). (c) Merge image of (a) and (b) with nuclei stained blue (DAPI). (d) to (f) A consecutive cryosection stained with isotype control MAbs. (d) No specific signal detected in palatine tonsil crypt epithelial cells with isotype control MAb TRT3 (green, anti-turkey rhinotracheitis virus). (e) No specific signal detected with isotype control MAb AV29 (gray, anti-chicken antigen). (f) Merge image of (d) and (e) with nuclei stained blue (DAPI). (d) to (f) Green and gray fluorescence in the adjacent germinal centre is due to autofluorescence associated with bovine germinal centres. Scale bars represent 100 μm .

2.4.2. Laser capture microdissection

2.4.2.1. Detecting FMDV genome

The ability to detect FMDV genome in laser microdissected tissue samples by rRT-PCR was initially evaluated using tongue epithelium cryosections harvested from cattle 3 days post-intradermolingual challenge (n = 4 animals) and from control cattle (n = 2). FMDV genome was detected consistently in epithelium samples laser dissected from the edge of FMDV lesions (n = 8 samples). Ct values ranged from 23.64 to 28.68. No signal was detected in the control tissue samples (n = 8) after 50 cycles.

2.4.2.2. Quantifying 28s rRNA

The ability to detect 28s rRNA was initially validated on PBMC (section 2.3.3.1) and laser microdissected mandibular lymph node and palatine tonsil samples. A dilution series of 5×10^4 to 5×10^1 PBMC were analysed in triplicate by rRT-PCR (Oleksiewicz *et al.*, 2001), approximately 100 PBMC contain 10^8 copies of 28s rRNA.

2.4.2.3. Tissue areas targeted for laser capture microdissection

The germinal centres and epithelium of the dorsal soft palates and pharyngeal tonsils (Liebler-Tenorio and Pabst, 2006) were targeted for LCM (Figure 10). The germinal centres, interfollicular regions, glandular epithelium and crypt epithelium of the palatine tonsils were targeted for LCM (Figure 11). The germinal centres and

interfollicular regions of the mandibular and lateral retropharyngeal lymph nodes were targeted for microdissection (Figure 12). The germinal centres and non-germinal centre regions of the splenic white pulp were targeted for LCM (Figure 12). Three replicates of the different tissue regions (germinal centres, epithelium etc) each containing six microdissected samples were collected from each tissue for RNA extraction.

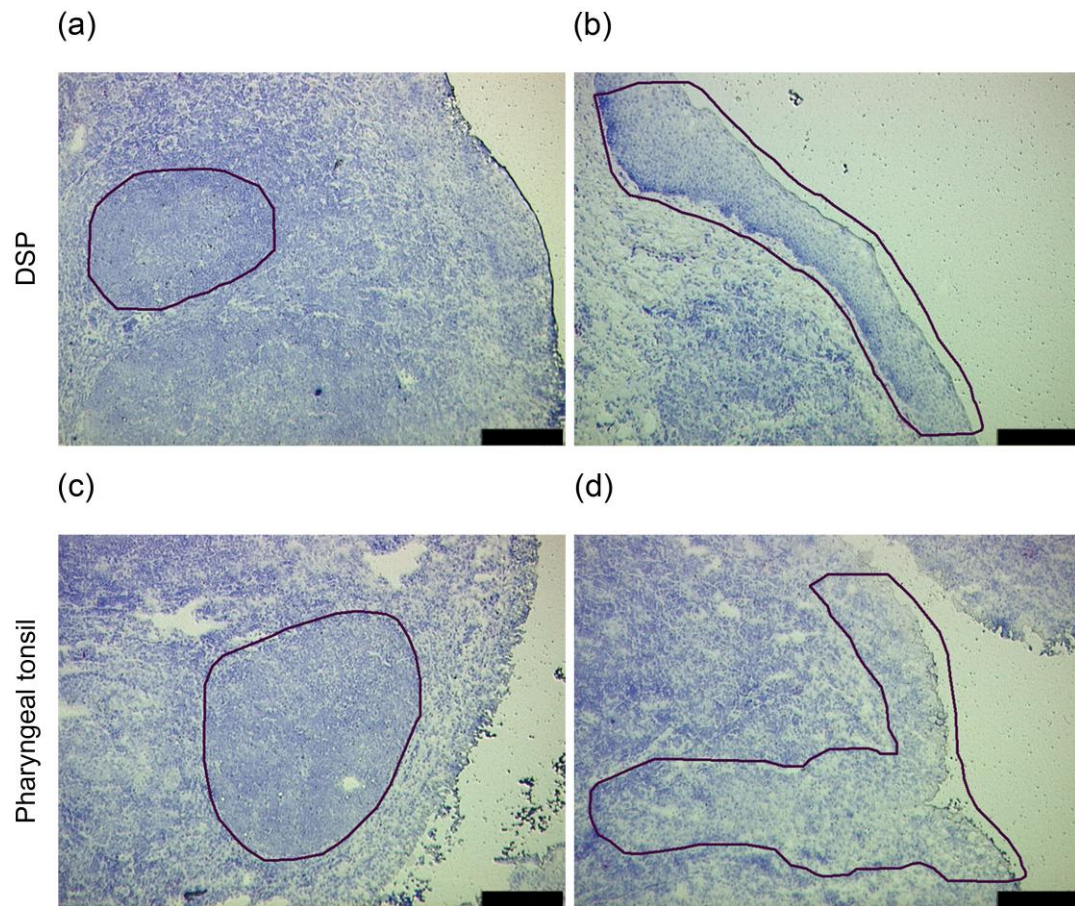


Figure 10. Regions of the dorsal soft palate and pharyngeal tonsil targeted for LCM. Dorsal soft palate (DSP) and pharyngeal tonsil cryosections stained with toluidine blue highlighting regions targeted during LCM. (a) Dorsal soft palate germinal centre and (b) epithelium targeted for LCM. (c) Pharyngeal tonsil germinal centre and (d) epithelium targeted for LCM. Scale bars represent 200µm.

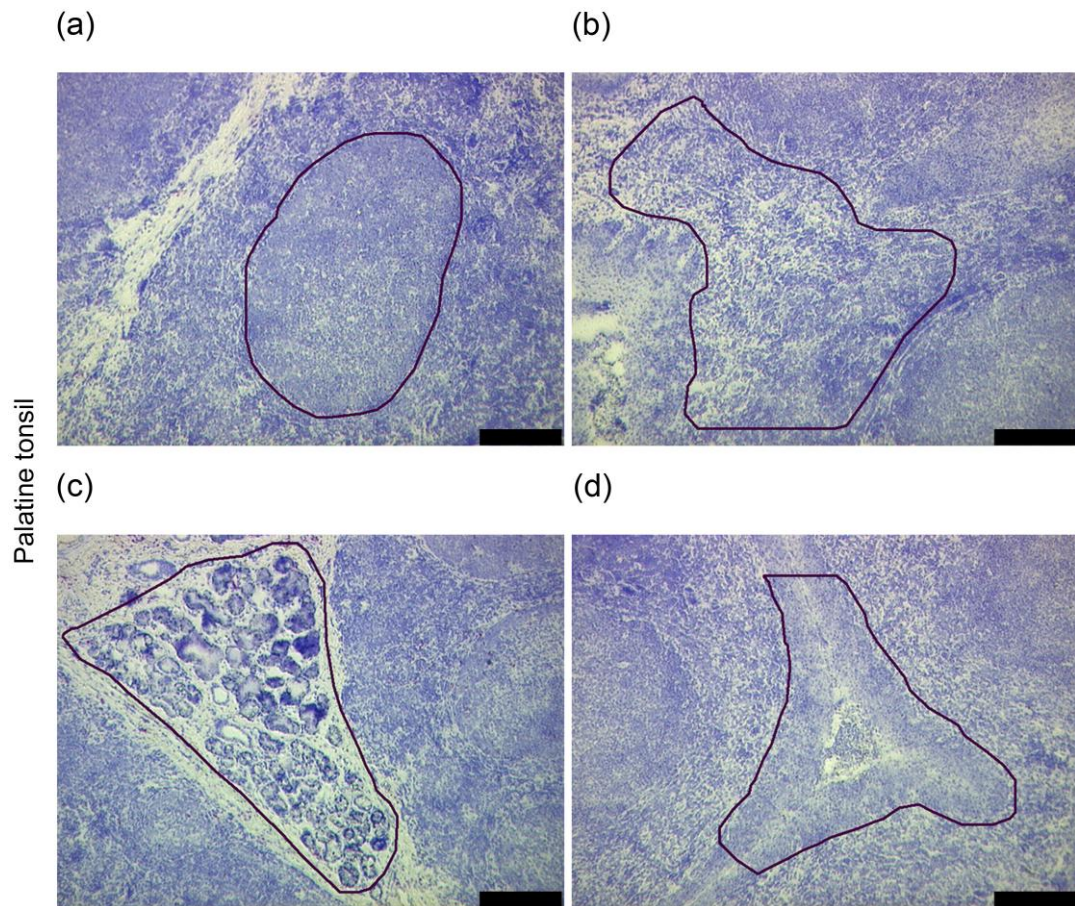


Figure 11. Regions of the palatine tonsil targeted for LCM.

Palatine tonsil cryosection stained with toluidine blue highlighting regions targeted during LCM. (a) Germinal centre, (b) interfollicular region, (c) glandular epithelium and (d) crypt epithelium targeted for LCM. Scale bars represent 200µm.

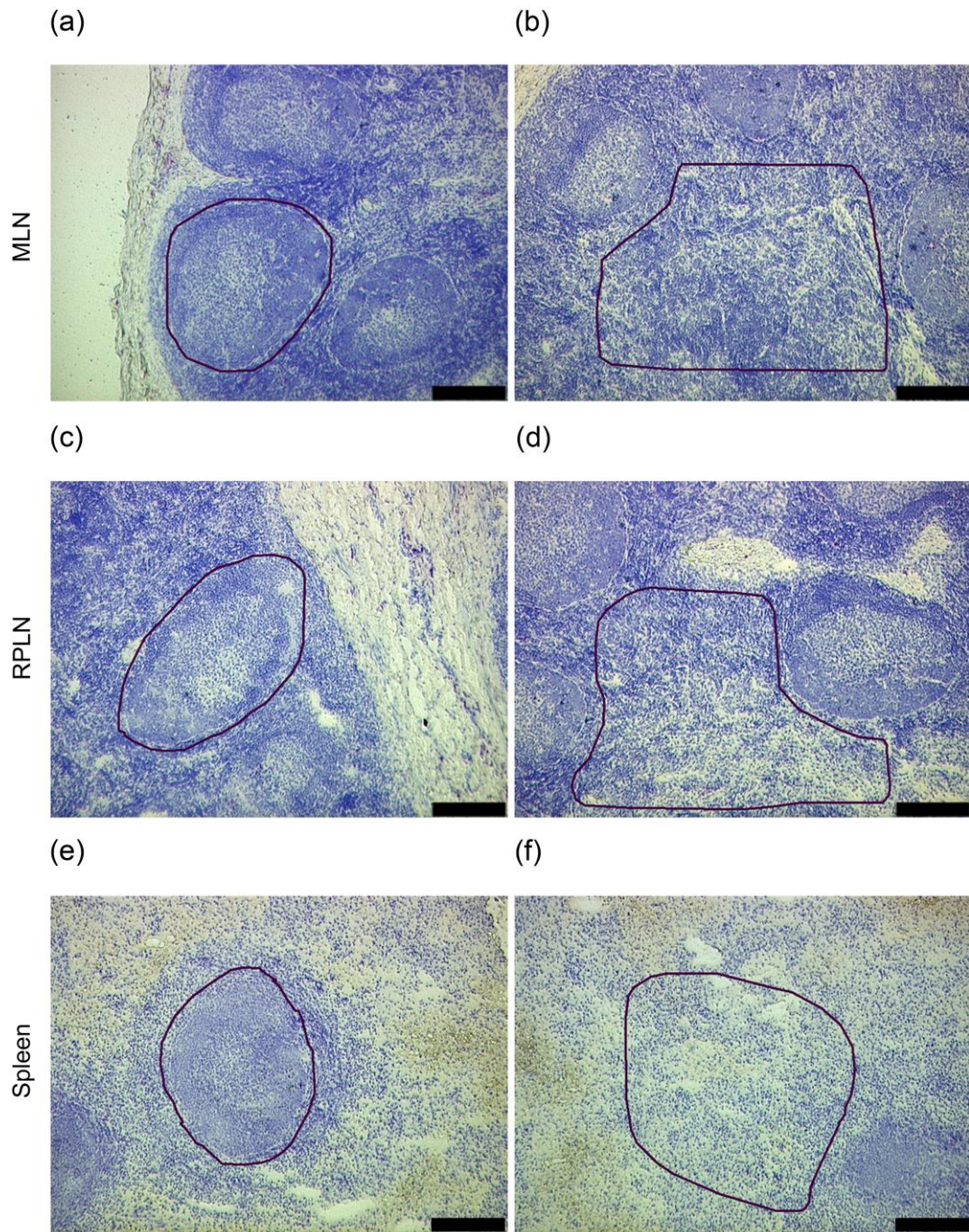


Figure 12. Regions of the mandibular lymph node, lateral retropharyngeal lymph node and spleen targeted for LCM. Mandibular lymph node (MLN), lateral retropharyngeal lymph node (RPLN) and spleen cryosections stained with toluidine blue highlighting regions targeted during LCM. (a) Mandibular lymph node germinal centre and (b) interfollicular region targeted for LCM. (c) Lateral retropharyngeal lymph node germinal centre and (d) interfollicular region targeted for LCM. (e) Germinal centre and (f) non-germinal centre regions of the splenic white pulp targeted for LCM. Scale bars represent 200µm.

2.4.2.4. Analysis of laser capture microdissected samples collected from animals 38 days post-contact infection

Tissues harvested from four cattle 38 days post-contact exposure to FMDV O UKG 34/2001 were selected for LCM (section 2.4.2.3). Probang samples collected at post-mortem were confirmed negative for FMDV by virus isolation and rRT-PCR.

FMDV genome and 28s rRNA were quantified by rRT-PCR analysis of laser microdissected samples. FMDV genome was detected consistently within the germinal centre samples obtained by LCM (Table 2, Figure 13 to Figure 18). No FMDV genome was detected in the epithelium of the dorsal soft palates and pharyngeal tonsils (Figure 13 and Figure 14). No FMDV genome was detected in the crypt epithelium, glandular epithelium and interfollicular regions of the palatine tonsils or the interfollicular regions of the mandibular lymph nodes and lateral retropharyngeal lymph nodes (Figure 15 to Figure 17). No FMDV genome was detected in the non-germinal centre regions of the splenic white pulp (Figure 18). No FMDV genome could be detected in germinal centre samples obtained by LCM from non-infected control animals. The R squared values (assessment of the fit of the standard curve line to the data points) ranged from 0.992 to 0.999 for the FMDV quantitative rRT-PCR reactions and from 0.998 to 0.999 for the 28s rRNA quantitative rRT-PCR reactions. The efficiency of the FMDV reactions ranged from 87.2 to 108.4% and for the 28s rRNA reactions from 86.3 to 93.3%. The number of copies of 28s rRNA per each PCR reaction are summarised in Figure 19. There was no statistically significant association between FMDV genome copies expressed as FMDV copies per 10^8 copies of 28s rRNA and amount of 28s rRNA per reaction ($P = 0.206$; ANOVA, general linear model). There was a statistically significant

association between the quantity of FMDV genome present in germinal centre samples and the type of tissue ($P = 0.0039$, Fisher's exact test). Significantly more FMDV genome copies per 10^8 copies of 28s rRNA were detected in replicates of six germinal centres from mandibular lymph nodes, compared to similar replicates harvested from other tissue (Mandibular lymph node compared to lateral retropharyngeal lymph node [$P = 0.0014$], mandibular lymph node compared to palatine tonsil [$P = 0.0376$], mandibular lymph node compared to pharyngeal tonsil [$P = 0.0392$] and mandibular lymph node compared to dorsal soft palate [$P = 0.0148$]; ANOVA, Tukey simultaneous test). The spleen samples were not included in the statistical analysis.

Table 2. Laser microdissected GC samples processed by quantitative rRT-PCR to detect FMDV.

Tissue*	Number of positive replicates	Number of negative replicates	Threshold cycle values of positive replicates**
DSP	9	3	38.74 to 46.24
Pharyngeal	6	6	36.76 to 40.22
Palatine tonsils	7	5	35.73 to 39.92
RPLN	12	0	34.68 to 37.01
MLN	12	0	35.64 to 40.03
Spleen	4	8	40.77 to 45.74

* 38 days post-contact infection (n = 4 animals). Only germinal centre samples were found to contain FMDV genome after 50 cycles.

** by rRT-PCR to detect FMDV genome.

DSP = dorsal soft palate.

RPLN = lateral retropharyngeal lymph node.

MLN = mandibular lymph node.

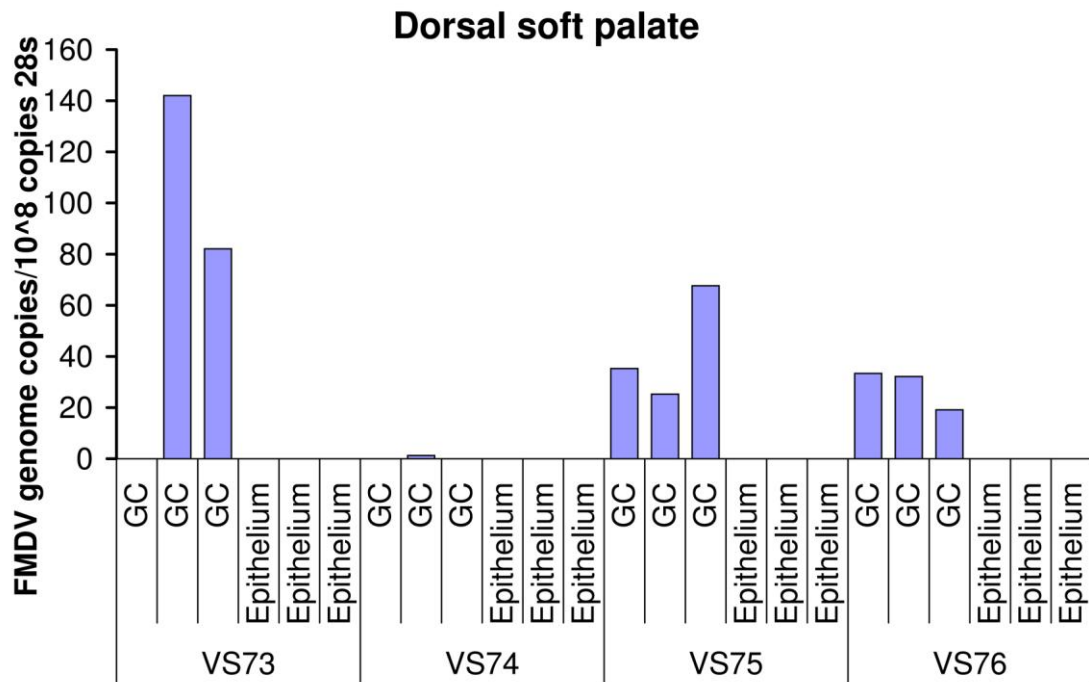


Figure 13. FMDV genome detected in laser microdissected dorsal soft palate samples. Dorsal soft palate samples analysed at 38 days post-contact infection by LCM in combination with quantitative rRT-PCR to detect FMDV genome. FMDV genome was restricted to germinal centre (GC) samples (n = 4 animals, each bar represents 6 microdissected samples). No fluorescent signal above threshold was detected in epithelial samples by rRT-PCR after 50 cycles.

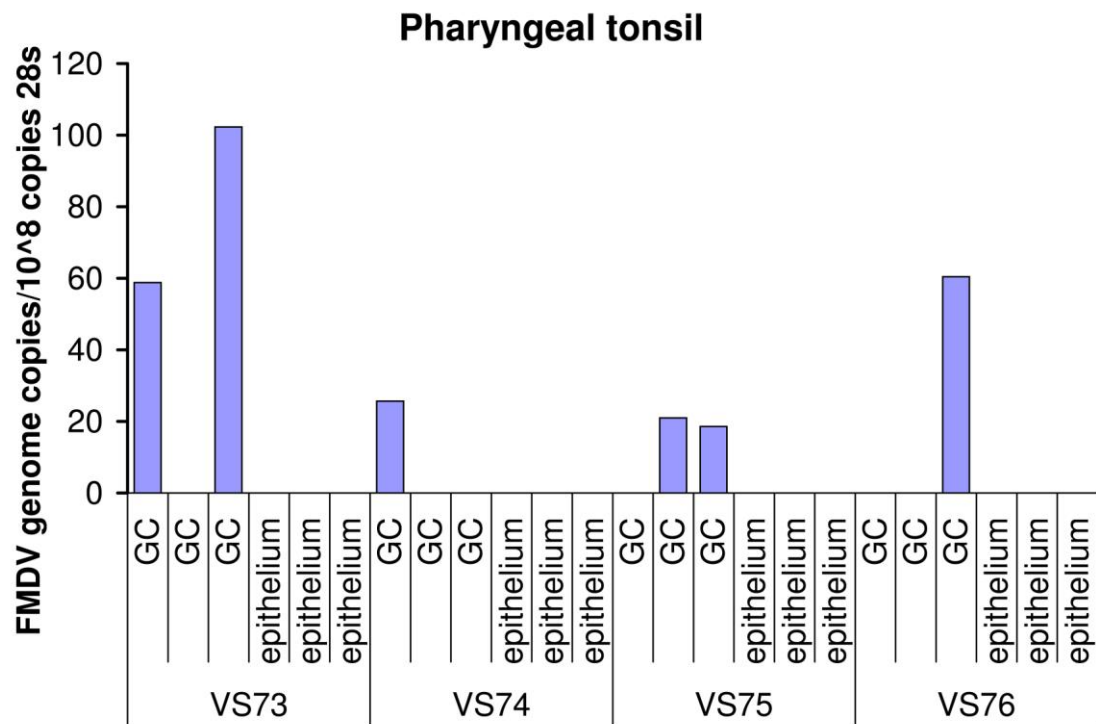


Figure 14. FMDV genome detected in laser microdissected pharyngeal tonsil samples. Pharyngeal tonsil samples analysed at 38 days post-contact infection by LCM in combination with quantitative rRT-PCR to detect FMDV genome. FMDV genome was restricted to germinal centre (GC) samples (n = 4 animals, each bar represents 6 microdissected samples). No fluorescent signal above threshold was detected in epithelial samples by rRT-PCR after 50 cycles.

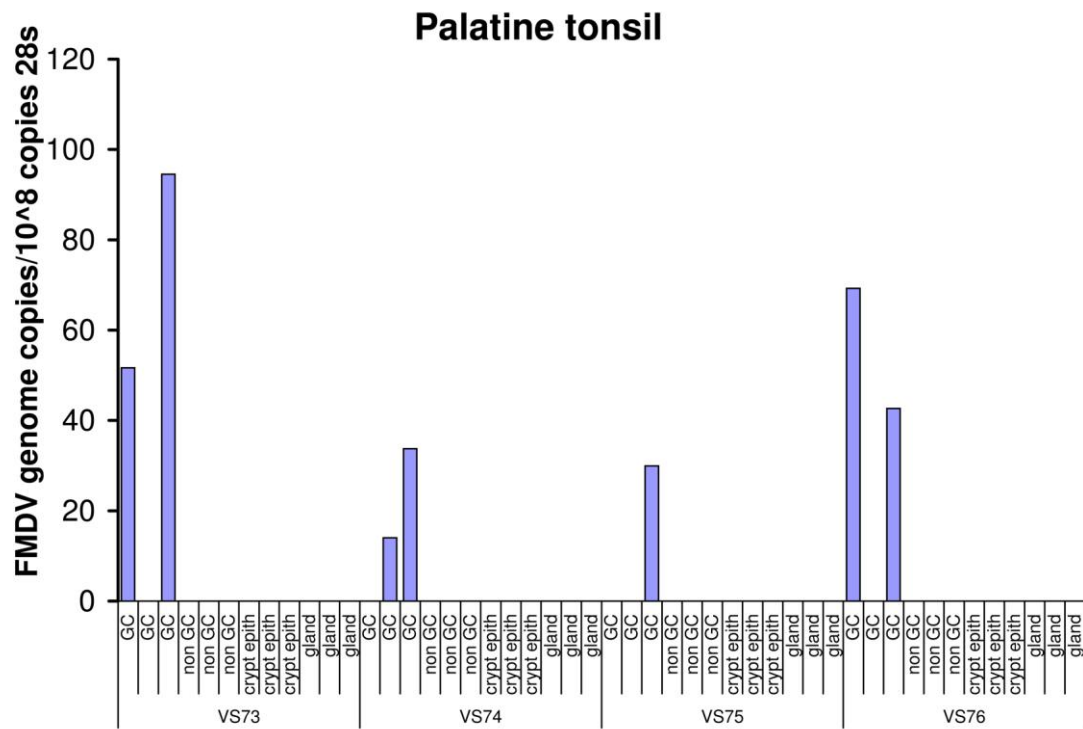


Figure 15. FMDV genome detected in laser microdissected palatine tonsil samples.

Palatine tonsil samples analysed at 38 days post-contact infection by LCM in combination with quantitative rRT-PCR to detect FMDV genome. FMDV genome was restricted to germinal centre (GC) samples ($n = 4$ animals, each bar represents 6 microdissected samples). No fluorescent signal above threshold was detected in interfollicular (non GC), crypt epithelium (crypt epith) or glandular epithelium (gland) samples by rRT-PCR after 50 cycles.

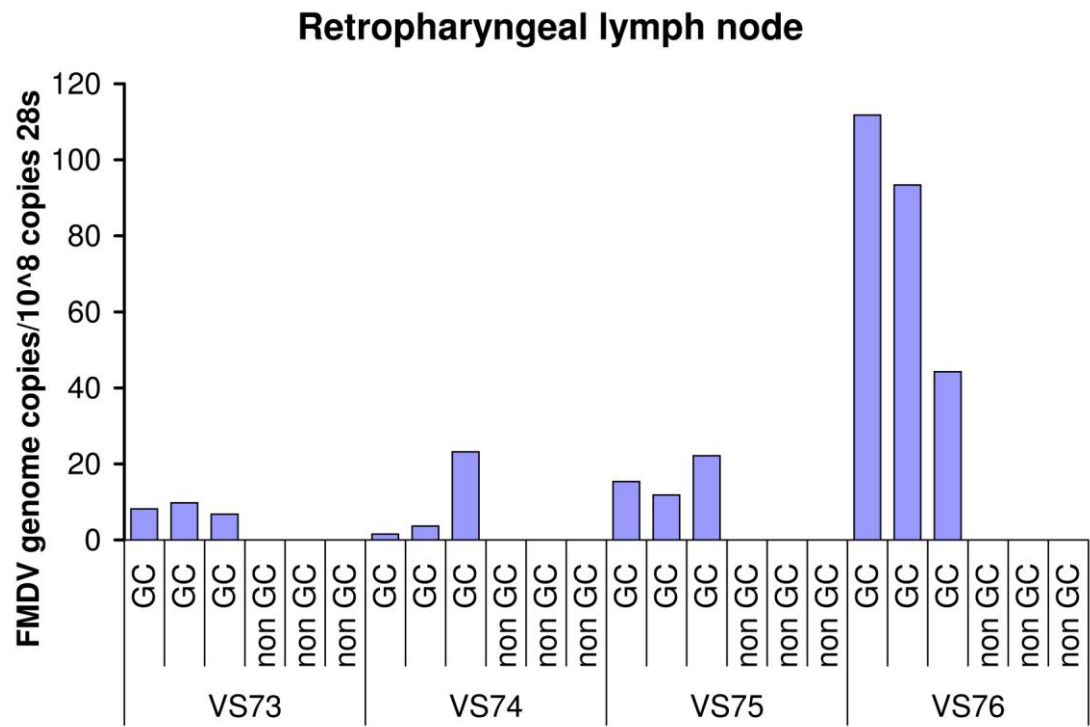


Figure 16. FMDV genome detected in lateral retropharyngeal lymph node samples. Lateral retropharyngeal lymph node samples analysed at 38 days post-contact infection by LCM in combination with quantitative rRT-PCR to detect FMDV genome. FMDV genome was restricted to germinal centre (GC) samples (n = 4 animals, each bar represents 6 microdissected samples). No fluorescent signal above threshold was detected in interfollicular (non GC) samples by rRT-PCR after 50 cycles.

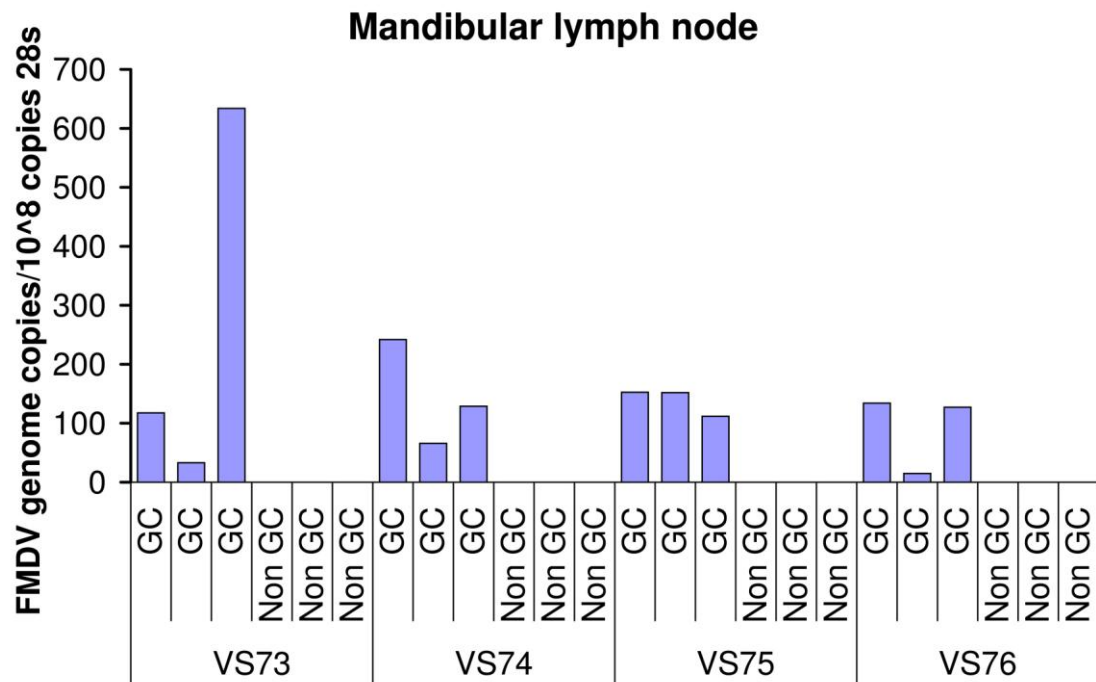


Figure 17. FMDV genome detected in laser microdissected mandibular lymph node samples. Mandibular lymph node samples analysed at 38 days post-contact infection by LCM in combination with quantitative rRT-PCR to detect FMDV genome. FMDV genome was restricted to germinal centre (GC) samples (n = 4 animals, each bar represents 6 microdissected samples). No fluorescent signal above threshold was detected in interfollicular (non GC) samples by rRT-PCR after 50 cycles.

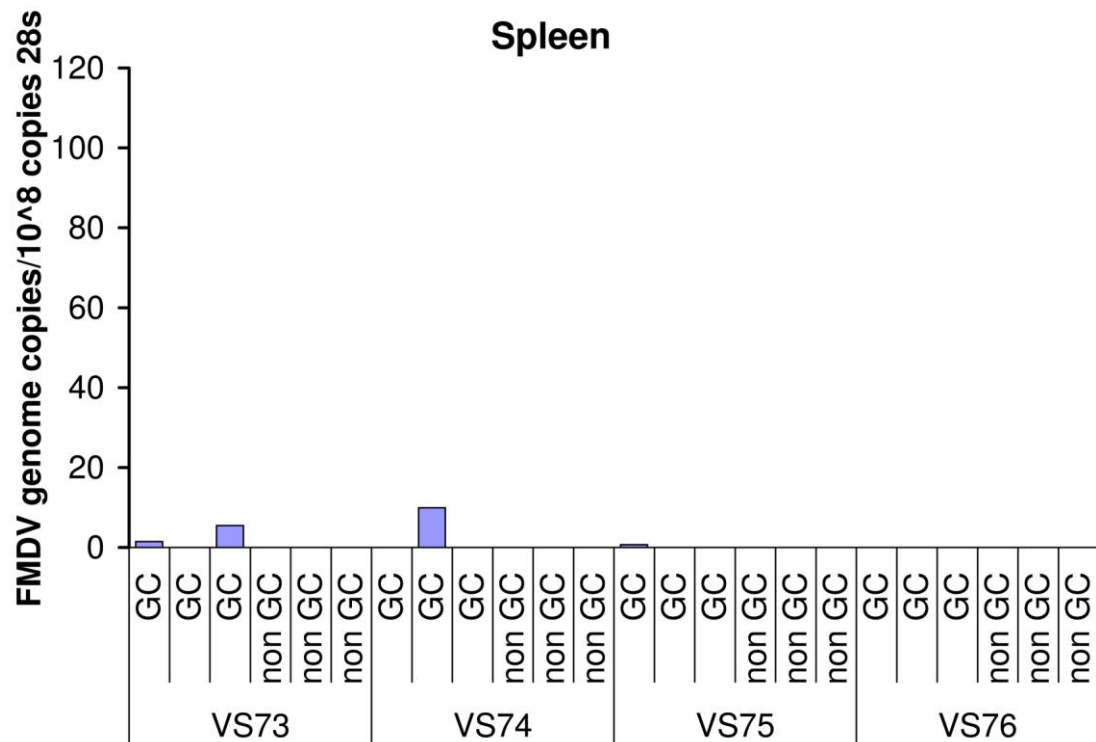


Figure 18. FMDV genome detected in laser microdissected splenic samples.

Splenic samples analysed 38 days post-contact infection by LCM in combination with quantitative rRT-PCR to detect FMDV genome. FMDV genome was restricted to germinal centre (GC) samples ($n = 4$ animals, each bar represents 6 microdissected samples). No fluorescent signal above threshold was detected in non-germinal centre (non-GC) samples of the splenic white pulp by rRT-PCR after 50 cycles.

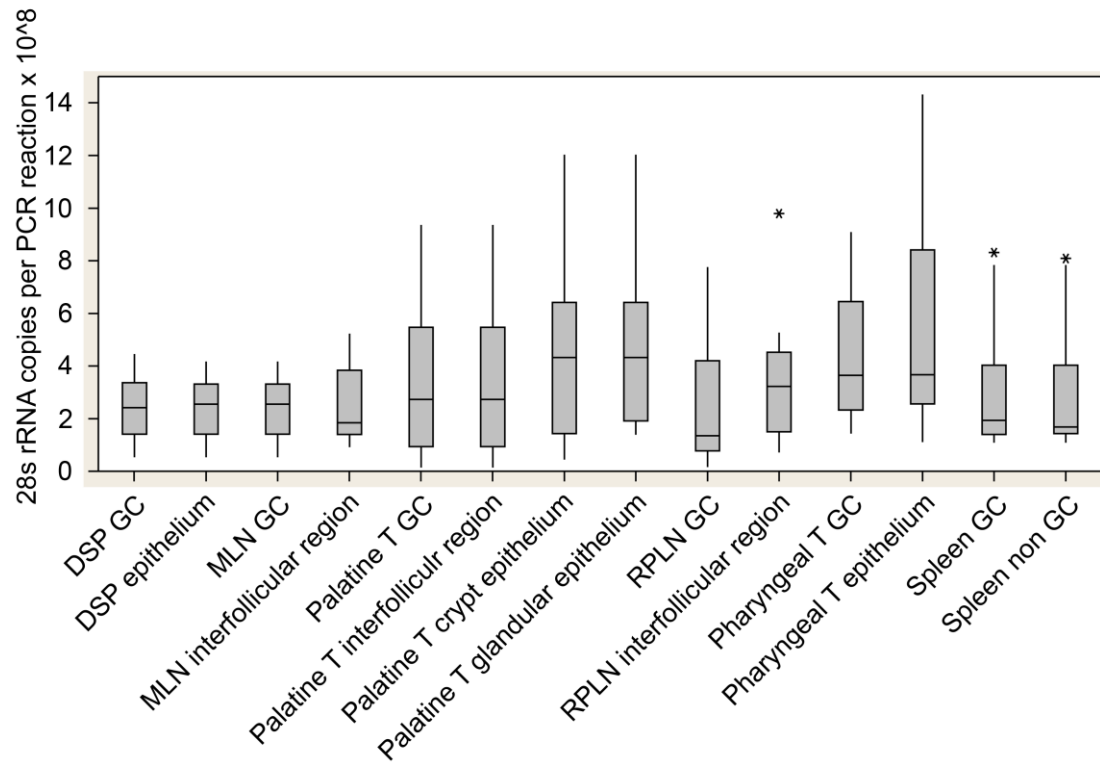


Figure 19. Copies of 28s rRNA per PCR reaction.

Boxplot summarising the number of copies of 28s rRNA per PCR reaction for each region of tissue sampled by LMD (n = 4 animals. Each plot depicts the data for 12 PCR reactions). GC = germinal centre. MLN = mandibular lymph node. Palatine T = palatine tonsil. RPLN = lateral retropharyngeal lymph node. Pharyngeal T = pharyngeal tonsil. Spleen non GC = non germinal centre region of the splenic white pulp. * = outlier values (value more than 1.5 × the interquartile range).

2.4.3. *In situ* hybridization

For *in situ* hybridization with unamplified conventional chromagenic detection, a dilution of 200ng/mL of RNA probe was found to be optimal. Optimal probe concentrations for TSA were tenfold lower than those used for unamplified chromagenic detection (Schaeren-Wiemers and Gerfin-Moser, 1993). Probe concentration is an essential parameter to consider for improving signal-to-noise ratio. Even at lower probe concentrations the signal remained equally intense, this observation is consistent with the hypothesis that in the absence of RNases, signal intensity is limited by the abundance of the target RNA rather than by the probe concentration.

The prepared hybridization buffer was replaced with the hybridization buffer supplied in the mRNA Locator *in situ* Hybridization Kits (Appendix 1). The buffers in this kit are optimised for use with radiolabelled RNA probes. DIG labelled probes and ³³P labelled probes behave with similar kinetics and may be used under similar hybridization conditions (Sambrook and Russel, 2001). RNase digestion significantly decreased non-specific background and was incorporated into the protocol even though there have been reports in the literature of loss of signal intensity and its use is probably dependent on the nature of the tissue under investigation (Yang *et al.*, 1999). Treatment with proteinase K did not offer any increase in signal or reduction in noise and was not used routinely (Wilkinson and Nieto, 1993).

2.4.3.1. Comparison of tyramide signal amplification with conventional chromagenic detection

In situ hybridization protocols were compared and optimised on consecutive pharyngeal tonsil cryosections harvested from an animal 38 days post-contact infection using IgG1 RNA probes (Figure 20). Using biotinyl-tyramide and streptavidin conjugated to alkaline phosphatase introduced an additional round of amplification which enhanced the signal intensity compared to conventional chromagenic detection.

2.4.3.2. Validation of FMDV 3D RNA probes

The FMDV 3D antisense RNA probe was validated on infected and mock infected BHK-21 cells (section 2.3.17.3). In addition, the probe was validated on frozen coronary band epithelium sections harvested from animals 4 days post-contact challenge and from non-infected control animals (Figure 21 and Figure 22). Despite the obvious signal obtained when detecting positive strand viral RNA in infected cells, it was difficult to detect negative strand viral RNA by *in situ* hybridization.

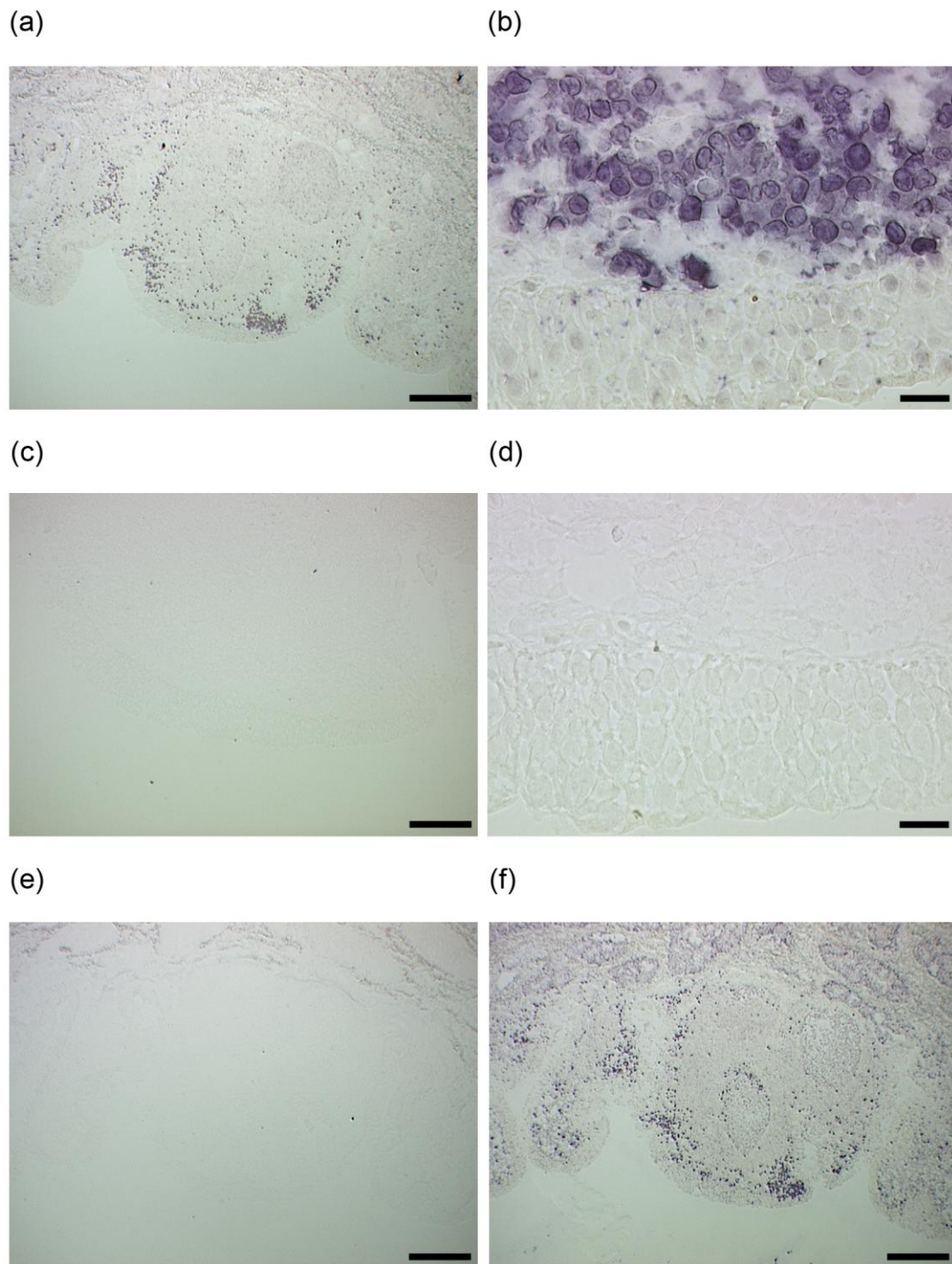


Figure 20. Comparison of tyramide signal amplification with conventional chromagenic detection.

Tyramide signal amplification and conventional chromagenic detection protocols were compared and optimised on consecutive pharyngeal tonsil cryosections, harvested from an animal 38 days post-contact infection, using IgG1 RNA probes. (a) and (b) IgG1 antisense probe detected with the tyramide signal amplification protocol, deposits of blue-black chromagen were detected in target cells with low background signal after developing for 2 minutes. (c) and (d) IgG1 antisense probe detected with conventional chromagenic protocol after developing for 2 minutes. No blue-black deposit associated with target cells. (e) Background signal with tyramide

signal amplification after developing for 30 minutes (IgG1 sense probe). (f) IgG1 antisense probe detected with conventional chromagenic protocol after developing for 30 minutes. Deposits of blue-black chromagen are associated with the target cells but high background signal makes the detection of rare mRNA difficult. Scale bars represent: (a), (e) and (f), 500 μ m; (b) and (d), 25 μ m; (c), 200 μ m.

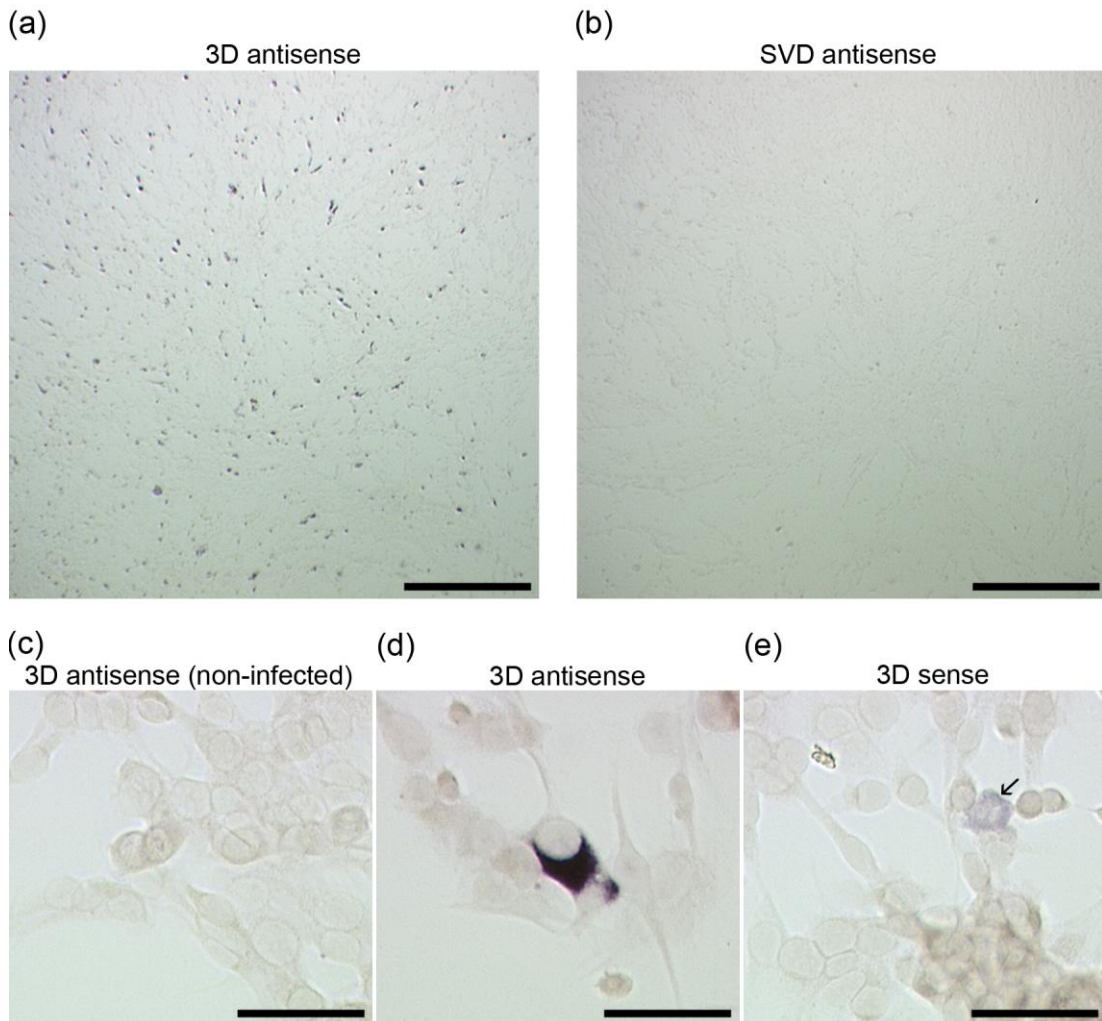


Figure 21. FMDV 3D RNA probe validation on infected and mock-infected BHK-21 cells.

(a) Positive signal after *in situ* hybridization with 3D antisense RNA probe on BHK-21 cells fixed 5 hours after FMDV O UKG 34/2001 infection at MOI 10. (b) Lack of specific signal on infected cells after *in situ* hybridization with swine vesicular disease (SVD) antisense probe. (c) Lack of specific signal on mock-infected cells after *in situ* hybridization with 3D antisense probe. (d) Positive, cytoplasmic blue-black chromagen deposit on infected cells after *in situ* hybridization with FMDV 3D antisense probe. (e) Faint blue-black chromagen deposit (arrow) after *in situ* hybridization with FMDV 3D sense probe. Scale bars represent: (a) and (b), 500 μ m; (c) to (e), 25 μ m.

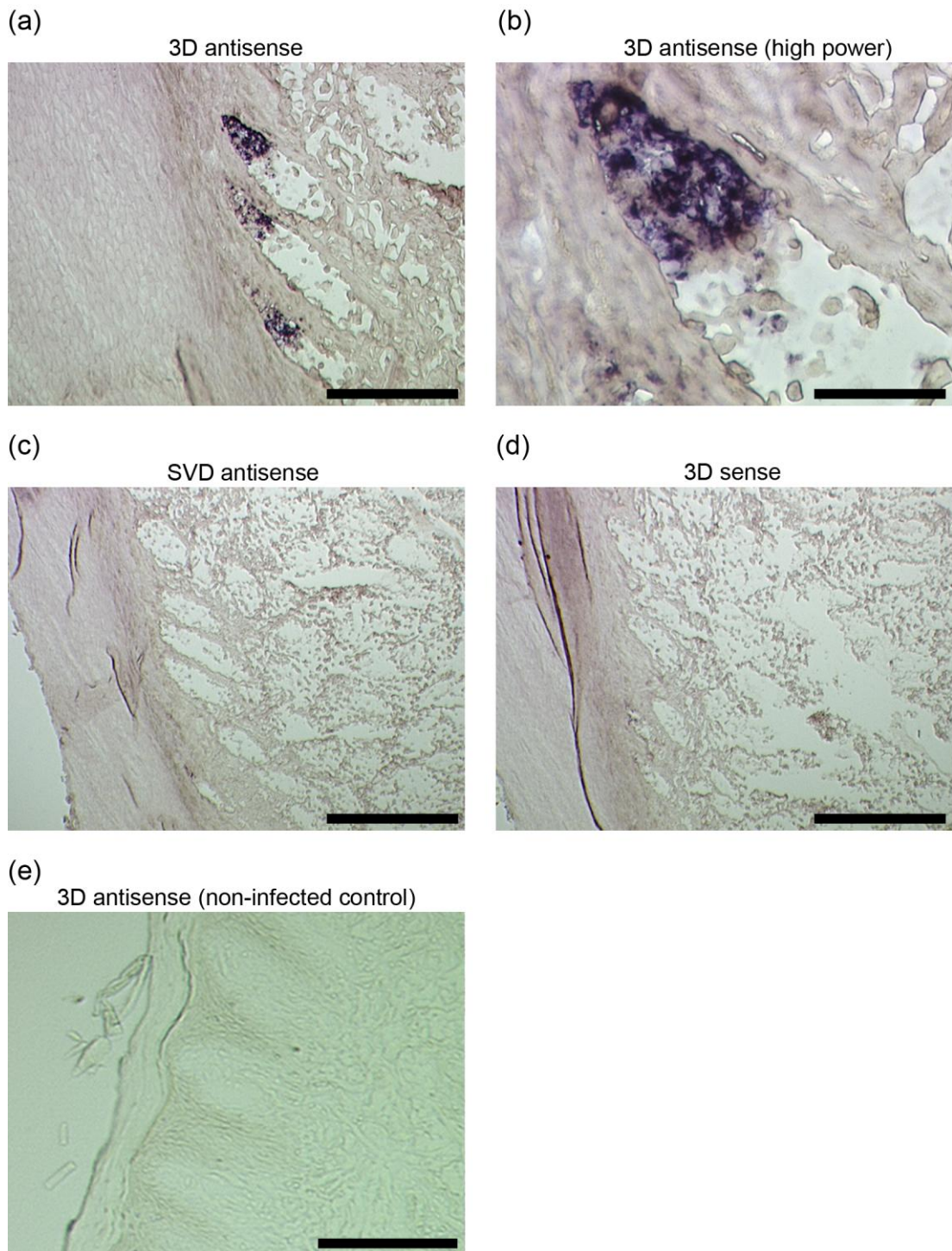


Figure 22. FMDV 3D RNA probe validation on infected and non-infected tissue.

The FMDV 3D RNA probes were validation on coronary band epithelium cryosections harvested from an animal 4 days post-contact infection and from a control animal. (a) and (b) Positive staining of coronary band epithelium harvested from an infected animal after *in situ* hybridization with FMDV 3D antisense RNA probe. (c) and (d) Lack of specific staining of infected coronary band epithelium after *in situ* hybridization with swine vesicular disease (SVD) antisense and FMDV 3D sense RNA probes respectively. (e) No staining was detected in non-infected

control tissue after *in situ* hybridization with FMDV 3D antisense RNA probe. Scale bars represent: (a), 200µm; (b), 50µm; (c) to (e), 50µm.

2.4.3.3. Analysis of tissue samples harvested 3 days post-infection

Tissue samples harvested 3 days post FMDV O UKG 34/2001 intradermolingual challenge were examined by *in situ* hybridization and tissue samples collected into RNAlater were analysed by quantitative rRT-PCR (Table 3). Clear staining, following *in situ* hybridization with FMDV 3D antisense RNA probe, was only observed in mandibular lymph node (Figure 23) and palatine tonsil sections as small, punctate isolated areas of blue-black chromagen deposition.

Table 3. Analysis of tissue samples harvested 3 days post-intradermolingual challenge.

Tissue	Number of animals sampled	Number of samples positive by <i>in situ</i> hybridization	Number of samples positive by rRT-PCR*	Range of genome copies (log copies/g tissue)
DSP	4	0	2	11.47-11.68
MLN	4	3	4	8.38-12.9
Palatine tonsil	4	2	4	9.55-12.96
Pharyngeal tonsil	4	0	2	11.97-12.71
RPLN	4	0	1	11.91
BLN	4	0	3	8.53-12.15

* Quantitative rRT-PCR analysis of tissue samples collected into RNAlater.

DSP = dorsal soft palate.

MLN = mandibular lymph node.

RPLN = lateral retropharyngeal lymph node.

BLN = bronchial lymph node.

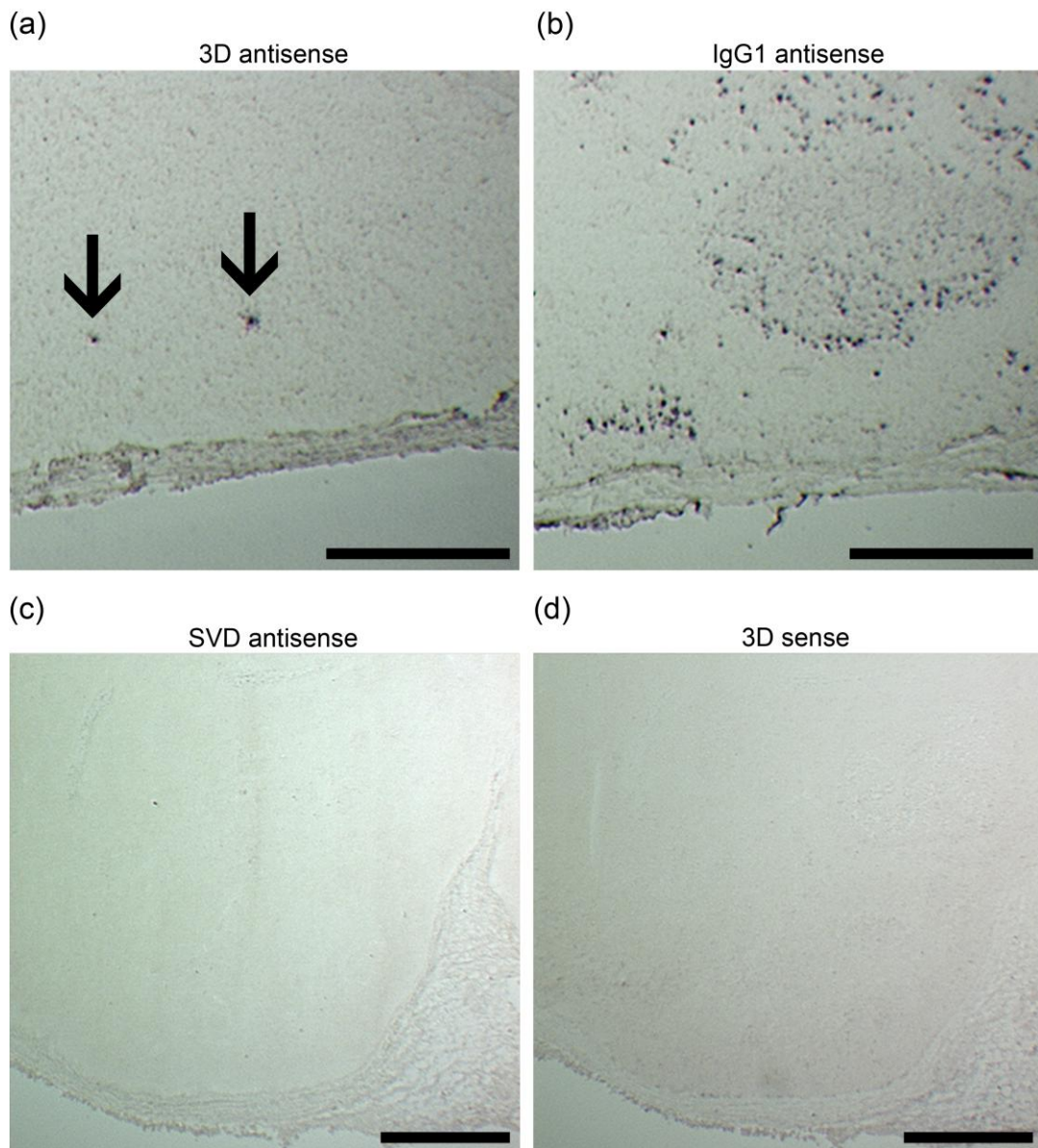


Figure 23. *In situ* hybridization analysis of mandibular lymph node cryosections harvested 3 days post-infection.

Consecutive mandibular lymph node cryosections harvested 3 days post-intradermolingual challenge. (a) Isolated areas of punctate staining (black arrows) after *in situ* hybridization with FMDV 3D antisense RNA probe. (b) Positive staining after *in situ* hybridization with IgG1 antisense RNA probe. (c) and (d) No staining was observed after *in situ* hybridization with SVD antisense or FMDV 3D RNA probes respectively. Scale bars represent 500µm.

2.4.3.4. Analysis of tissue samples harvested from 14 to 38 days post-contact infection

Tissue samples harvested from 14 to 38 days post FMDV O UKG 34/2001 contact infection were examined by *in situ* hybridization and tissue samples collected into RNAlater were analysed by quantitative rRT-PCR (Table 4). FMDV 3D RNA was identified by *in situ* hybridization in germinal centres of mandibular lymph node (Figure 24), lateral retropharyngeal lymph node (Figure 25) and palatine tonsil sections (Figure 26) but not in other compartments of these tissues.

Table 4. Analysis of tissue samples harvested from 14 to 38 days post-contact infection.

Tissue	Number of animals sampled	Number of samples positive by <i>in situ</i> hybridization	Number of samples positive by rRT-PCR*	Range of genome copies (log copies/g tissue)
DSP	10	0	2	9.34-10.32
MLN	10	4	8	6.32-11.5
Palatine tonsil	10	2	4	10.54-11.36
Pharyngeal tonsil	10	0	3	7.76-10.24
RPLN	10	1	2	7.5-10.3
BLN	10	0	0	0

* Quantitative rRT-PCR analysis of tissue samples collected into RNAlater.

DSP = dorsal soft palate.

MLN = mandibular lymph node.

RPLN = lateral retropharyngeal lymph node.

BLN = bronchial lymph node.

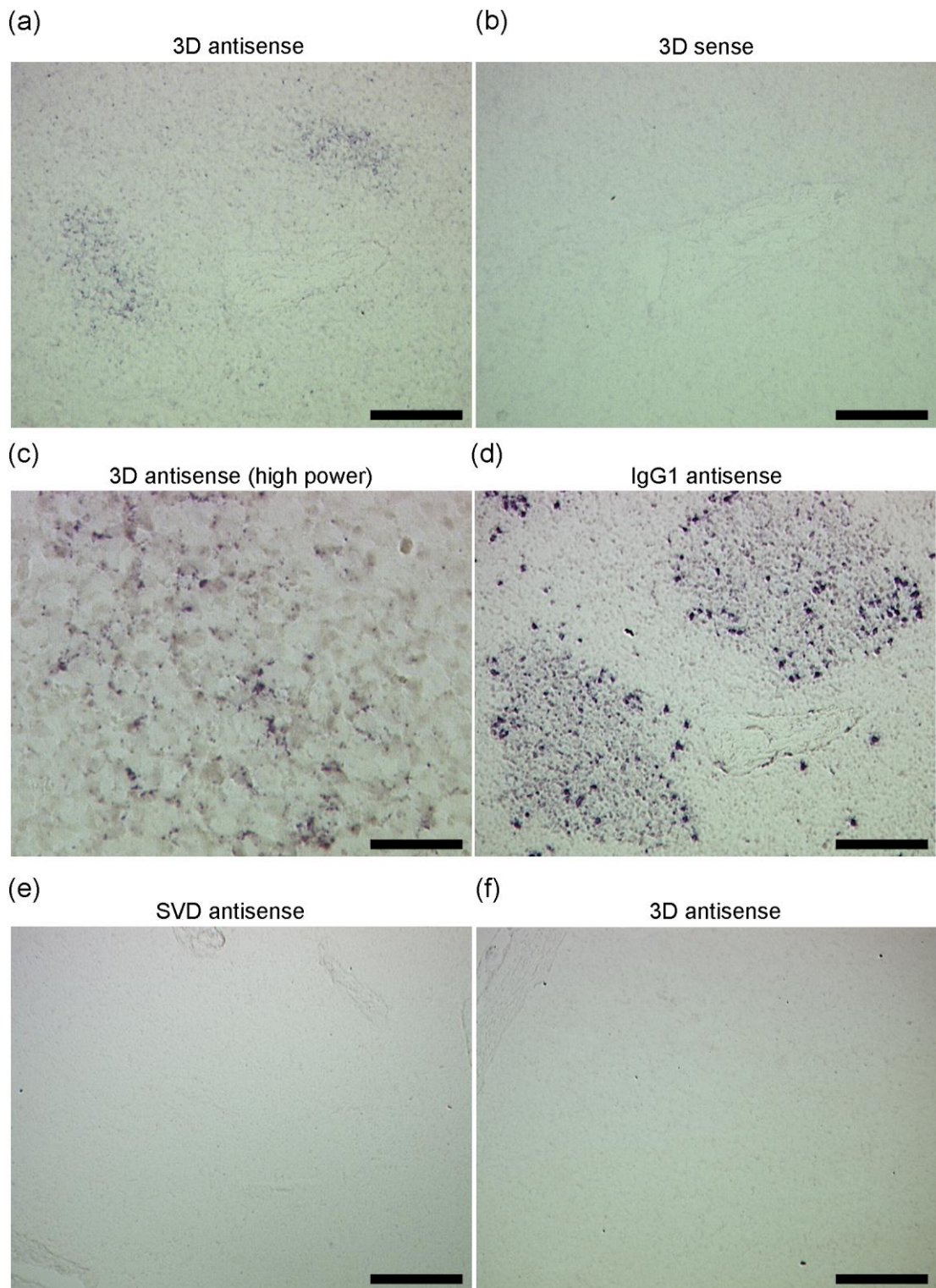


Figure 24. *In situ* hybridization analysis of mandibular lymph node cryosections harvested 38 days post-infection and from a non-infected control animal.

(a) to (e) Consecutive mandibular lymph node cryosections harvested 38 days post-contact infection. (a) FMDV 3D antisense RNA probe detecting sense FMDV 3D RNA. (b) Lack of staining after *in situ* hybridization with FMDV 3D sense RNA control probe. (c) Higher power image of staining associated with FMDV 3D antisense RNA probe. (d) Positive staining of IgG1 mRNA in germinal centre B cells

after *in situ* hybridization with IgG1 antisense RNA positive control probe. (e) Lack of staining after *in situ* hybridization with SVD antisense RNA control probe. (f) Lack of staining after *in situ* hybridization with FMDV 3D antisense RNA probe on a mandibular lymph node cryosection harvested from a non-infected control animal. Scale bars represent: (a), (b) and (d), 200 μ m; (c), 50 μ m; (e) and (f), 500 μ m.

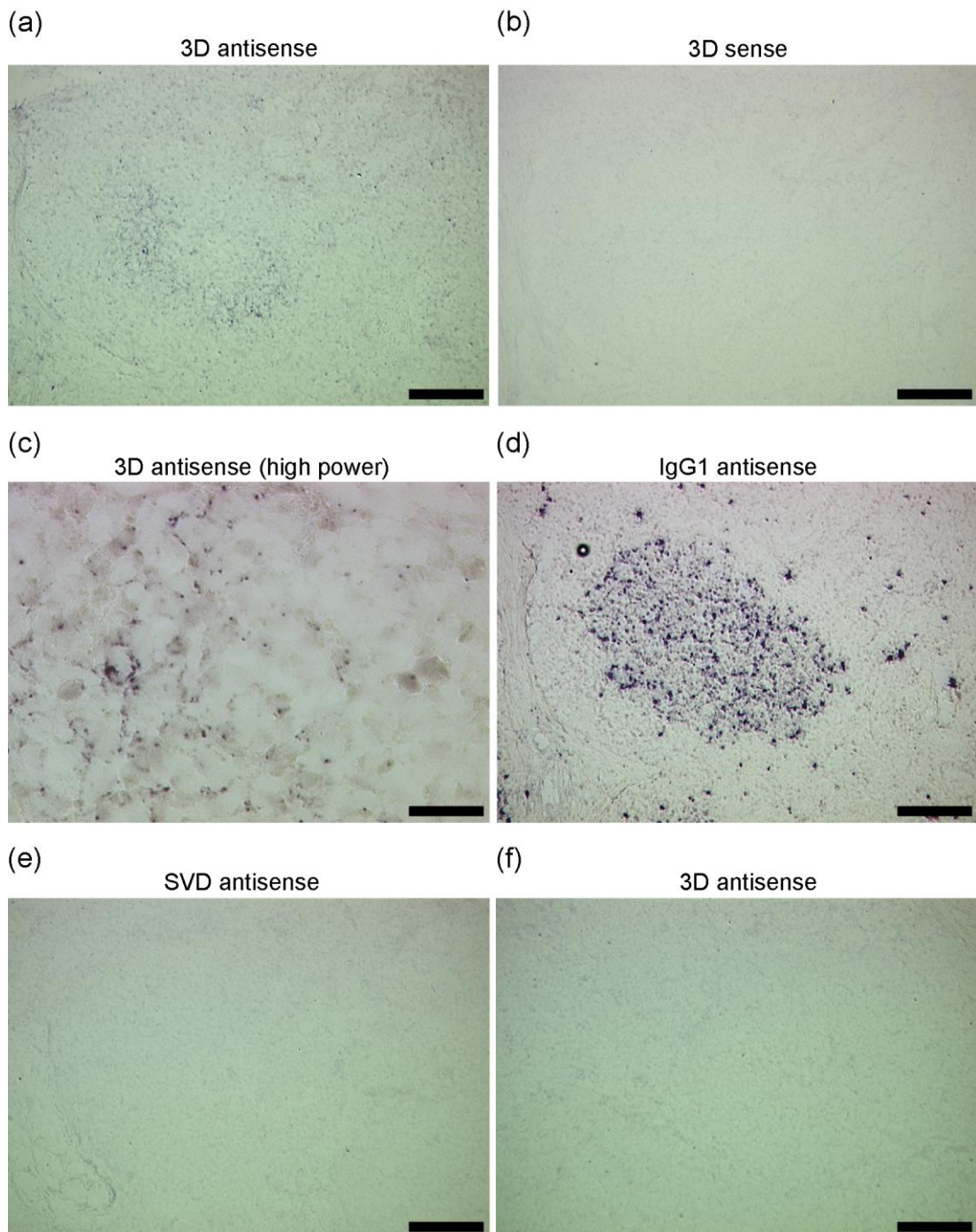


Figure 25. *In situ* hybridization analysis of lateral retropharyngeal lymph node cryosections harvested 22 days post-infection and from a non-infected control animal.

(a) to (e) Consecutive lateral retropharyngeal lymph node cryosections harvested 22 days post-contact infection. (a) FMDV 3D antisense RNA probe detecting sense

FMDV 3D RNA. (b) Lack of staining after *in situ* hybridization with FMDV 3D sense RNA control probe. (c) Higher power image of staining associated with FMDV 3D antisense RNA probe. (d) Positive staining of IgG1 mRNA in germinal centre B cells after *in situ* hybridization with IgG1 antisense RNA positive control probe. (e) Lack of staining after *in situ* hybridization with SVD antisense RNA control probe. (f) Lack of staining after *in situ* hybridization with FMDV 3D antisense RNA probe on a lateral retropharyngeal lymph node cryosection harvested from a non infected control animal. Scale bars represent: (a), (b) and (d), 200µm; (c), 50µm; (e) and (f), 500µm.

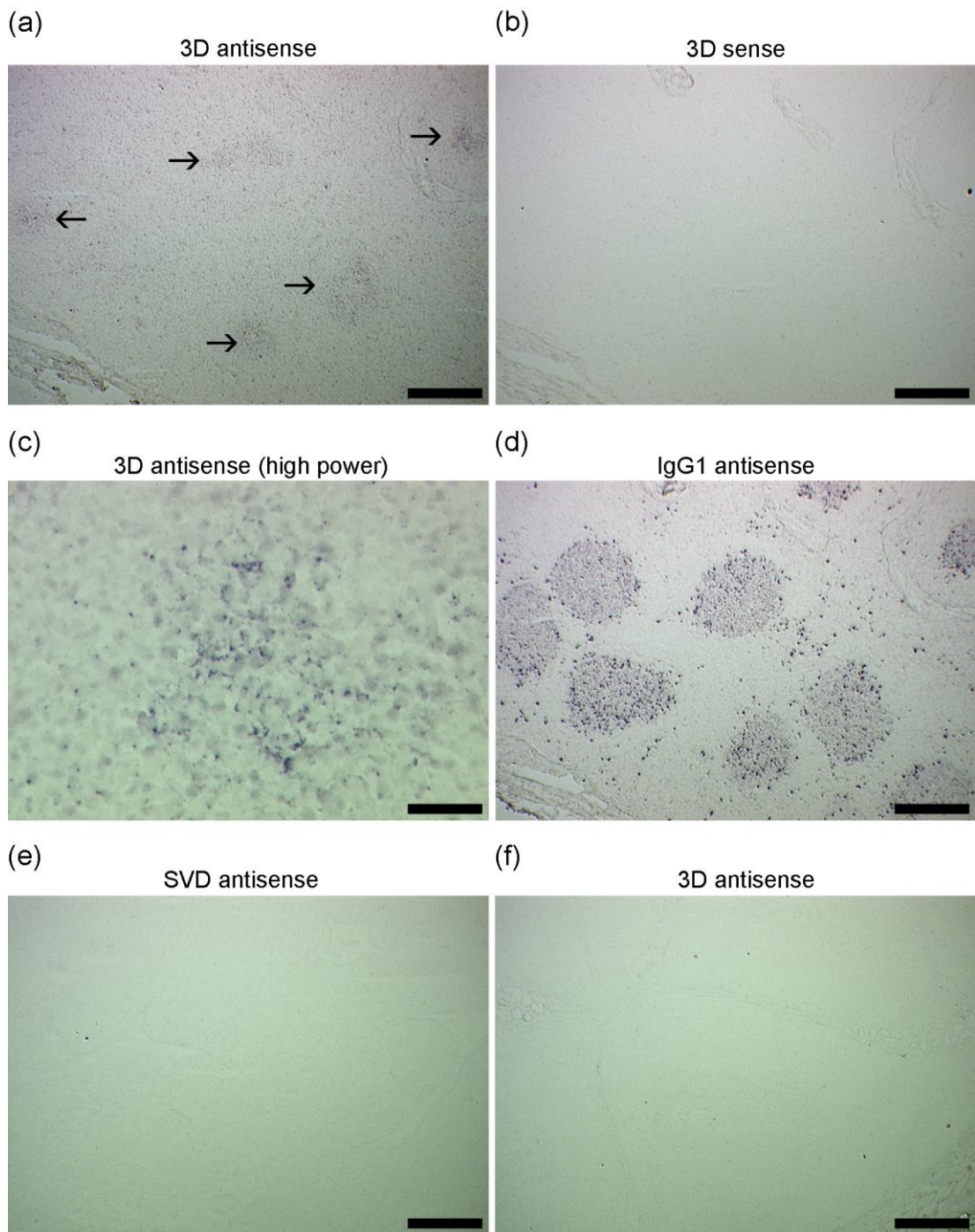


Figure 26. *In situ* hybridization analysis of palatine tonsil cryosections harvested 32 days post-infection and from a non-infected control animal.

(a) to (e) Palatine tonsil cryosections harvested 32 days post-contact infection. (a) FMDV 3D antisense RNA probe detecting sense FMDV 3D RNA (black arrows). (b) Lack of staining after *in situ* hybridization with FMDV 3D sense RNA control probe. (c) Higher power image of staining associated with FMDV 3D antisense RNA probe. (d) Positive staining of IgG1 mRNA in germinal centre B cells after *in situ* hybridization with IgG1 antisense RNA positive control probe. (e) Lack of staining after *in situ* hybridization with SVD antisense RNA control probe. (f) Lack of staining after *in situ* hybridization with FMDV 3D antisense RNA probe on a

Palatine tonsil cryosection harvested from a non-infected control animal. Scale bars represent: (a), (b) and (d), 200µm; (c), 50µm; (e) and (f), 500µm.

2.4.4. Immunofluorescence confocal microscopy

2.4.4.1. Selection of monoclonal antibodies specific for conformational, non-neutralising epitopes of the FMDV capsid

MAbs IB11, FC6, AD10 and BF8 (Table 1) were able to immunoprecipitate FMDV capsids, yet were unable to detect FMDV proteins by western blot and were non-neutralising (Juleff *et al.*, 2008). The MAbs readily detected virus in bovine tongue during acute FMDV O UKG 34/2001 infection (Figure 27 to Figure 31) and in virus infected BHK-21 cells (Figure 32).

2.4.4.2. Detecting FMDV immune complexes

MAb IB11 was able to detect immune complexed FMDV *in vitro* on the surface of paraformaldehyde fixed mouse fibroblast 3T3 cells (Appendix 1) expressing bovine CD32 (Figure 33).

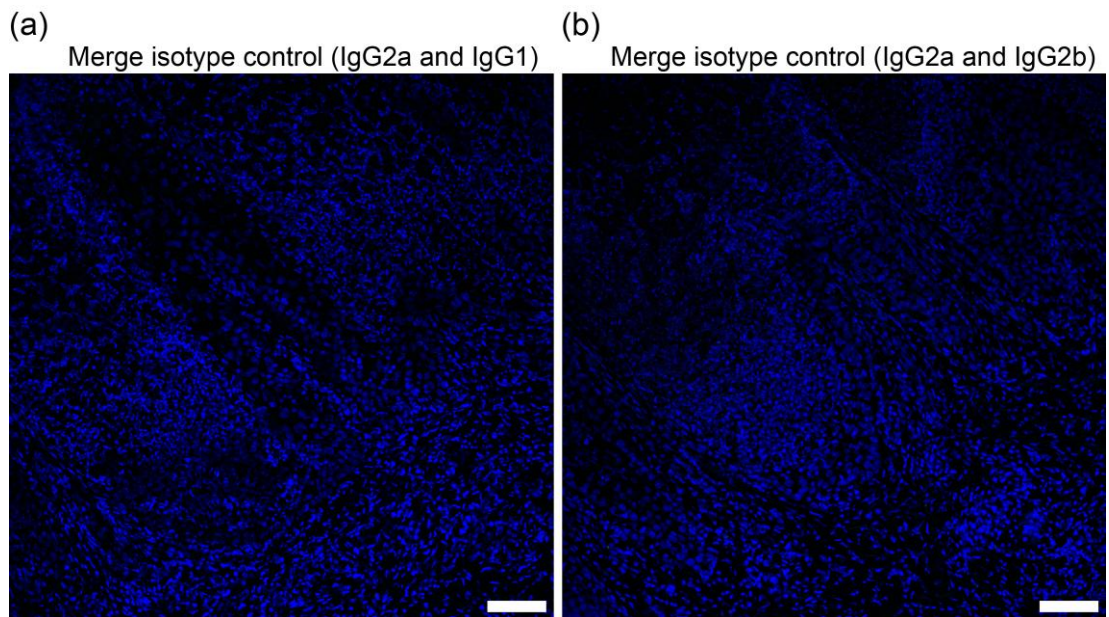


Figure 27. Infected tongue epithelium stained with isotype control antibodies.
 Infected tongue epithelium cryosections harvested 4 days post-contact challenge. (a) No signal was detected with isotype control MAb TRT3 (red, anti-turkey rhinotracheitis virus) or TRT1 (green, anti-turkey rhinotracheitis virus). (b) No signal was detected with isotype control MAb TRT3 (red) or AV29 (green, anti-chicken antigen). Nuclei stained blue (DAPI). Scale bars represent 80µm.

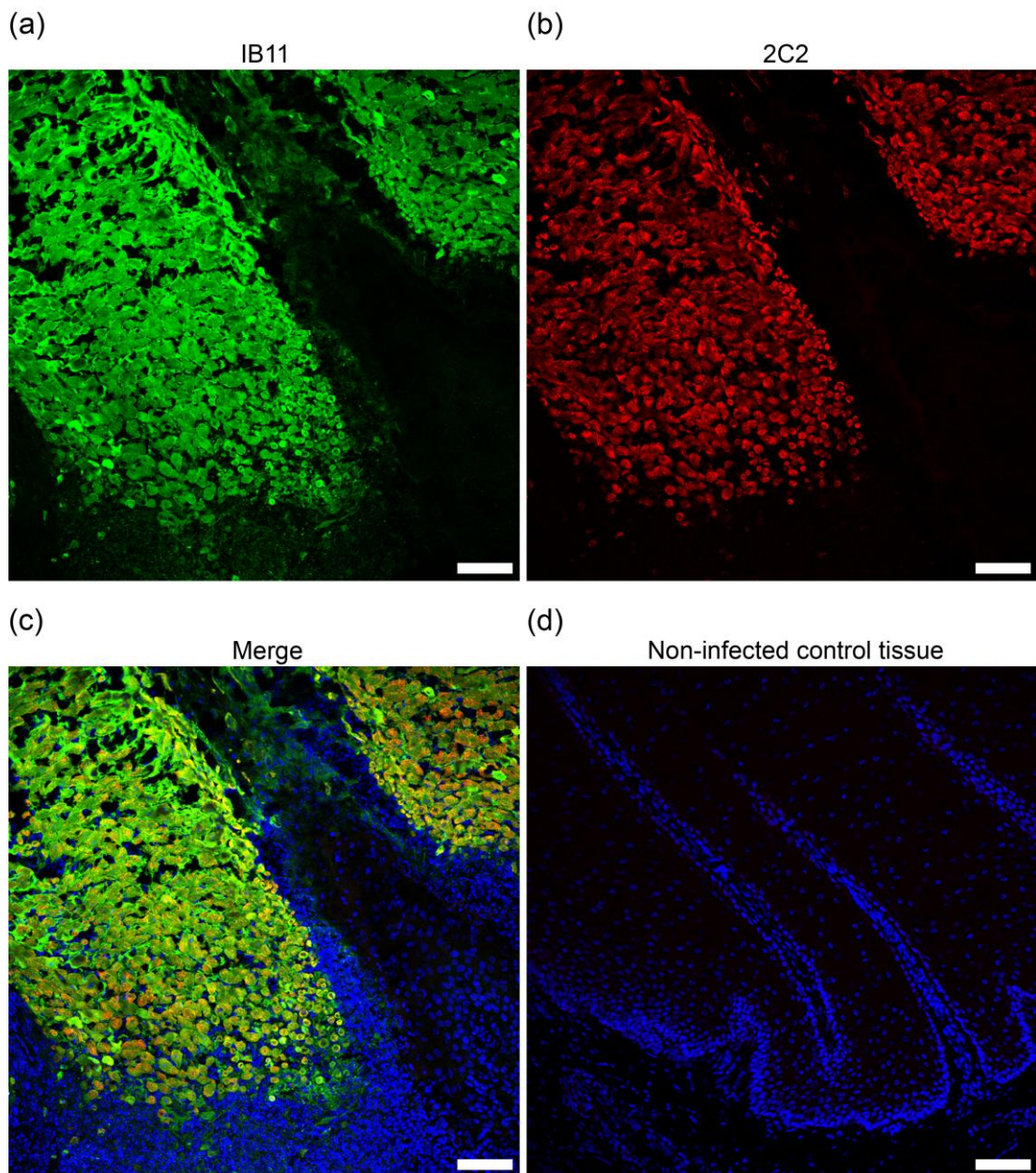


Figure 28. Infected and non-infected tongue epithelium stained with MAbs IB11 and 2C2. (a) to (c) Infected tongue epithelium cryosections harvested 4 days post-contact challenge. (a) FMDV capsids stained green (anti-FMDV capsid MAb IB11). (b) FMDV non-structural protein 3A stained red (anti-FMDV 3A MAb 2C2). (c) Merge image of (a) and (b) highlighting the co-localisation of FMDV capsid and 3A proteins. (d) No signal was detected with MAbs IB11 (green) or 2C2 (red) on non-infected control tissue. Nuclei stained blue (DAPI), scale bars represent 80µm.

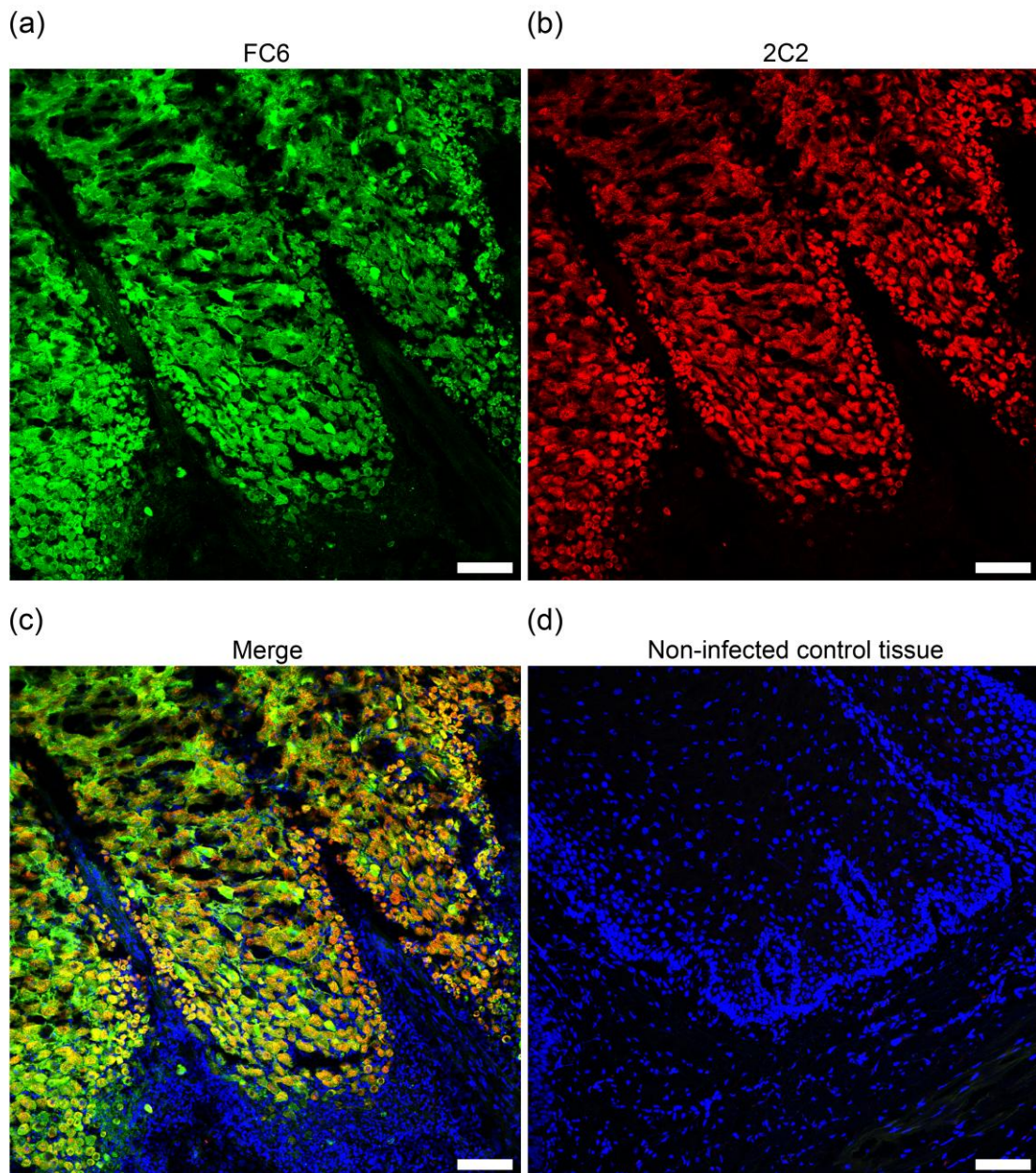


Figure 29. Infected and non-infected tongue epithelium stained with MAbs FC6 and 2C2. (a) to (c) Infected tongue epithelium cryosections harvested 4 days post-contact challenge (a) FMDV capsids stained green (anti-FMDV capsid MAb FC6). (b) FMDV non-structural protein 3A stained red (anti-FMDV 3A MAb 2C2). (c) Merge image of (a) and (b) highlighting the co-localisation of FMDV capsid and 3A proteins. (d) No signal was detected with MAbs FC6 (green) or 2C2 (red) on non-infected control tissue. Nuclei stained blue (DAPI), scale bars represent 80µm.

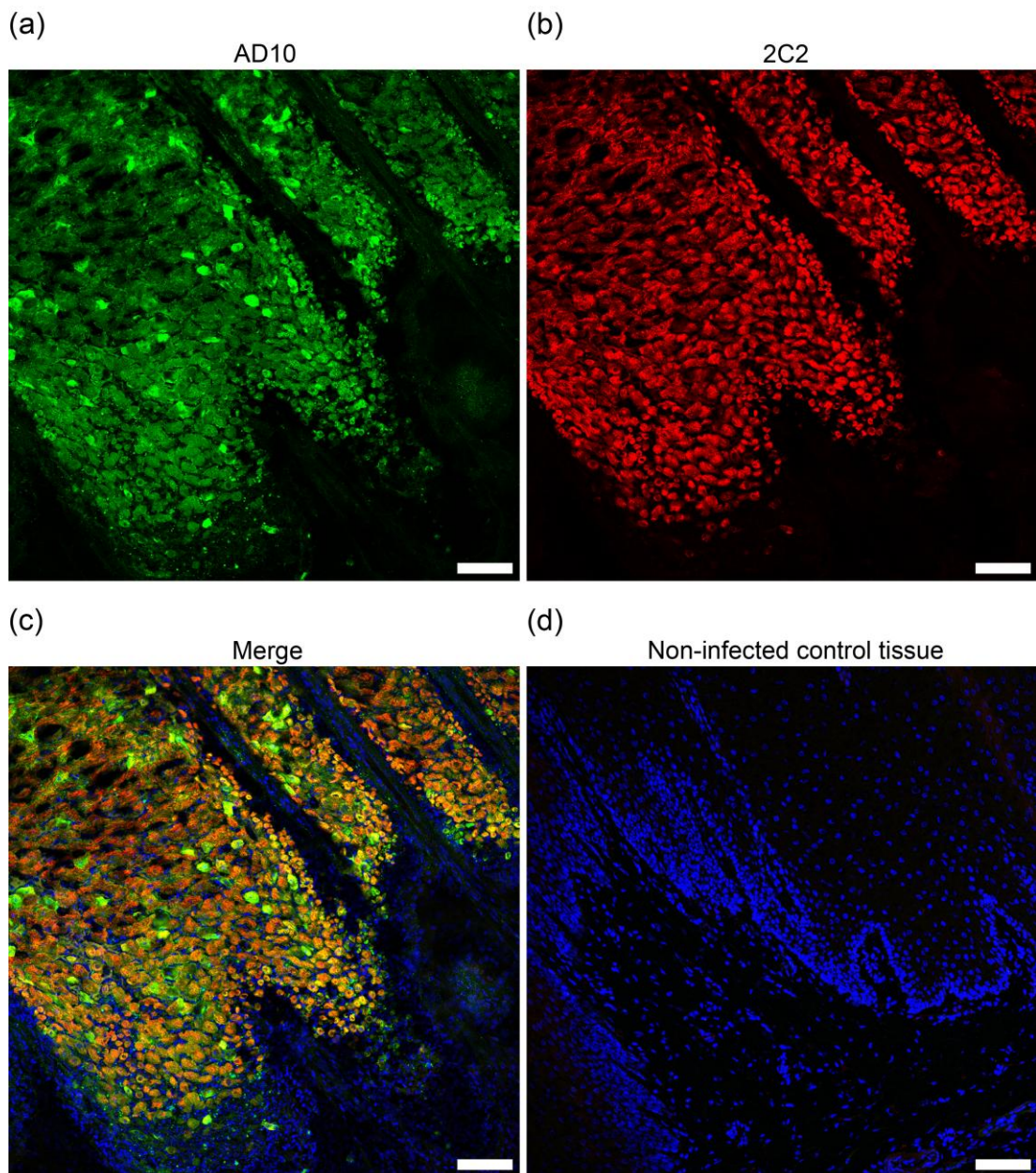


Figure 30. Infected and non-infected tongue epithelium stained with MAbs AD10 and 2C2. (a) to (c) Infected tongue epithelium cryosections harvested 4 days post-contact challenge. (a) FMDV capsids stained green (anti-FMDV capsid MAb AD10). (b) FMDV non-structural protein 3A stained red (anti-FMDV 3A MAb 2C2). (c) Merge image of (a) and (b) highlighting the co-localisation of FMDV capsid and 3A proteins. (d) No signal was detected with MAbs AD10 (green) or 2C2 (red) on non-infected control tissue. Nuclei stained blue (DAPI), scale bars represent 80µm.

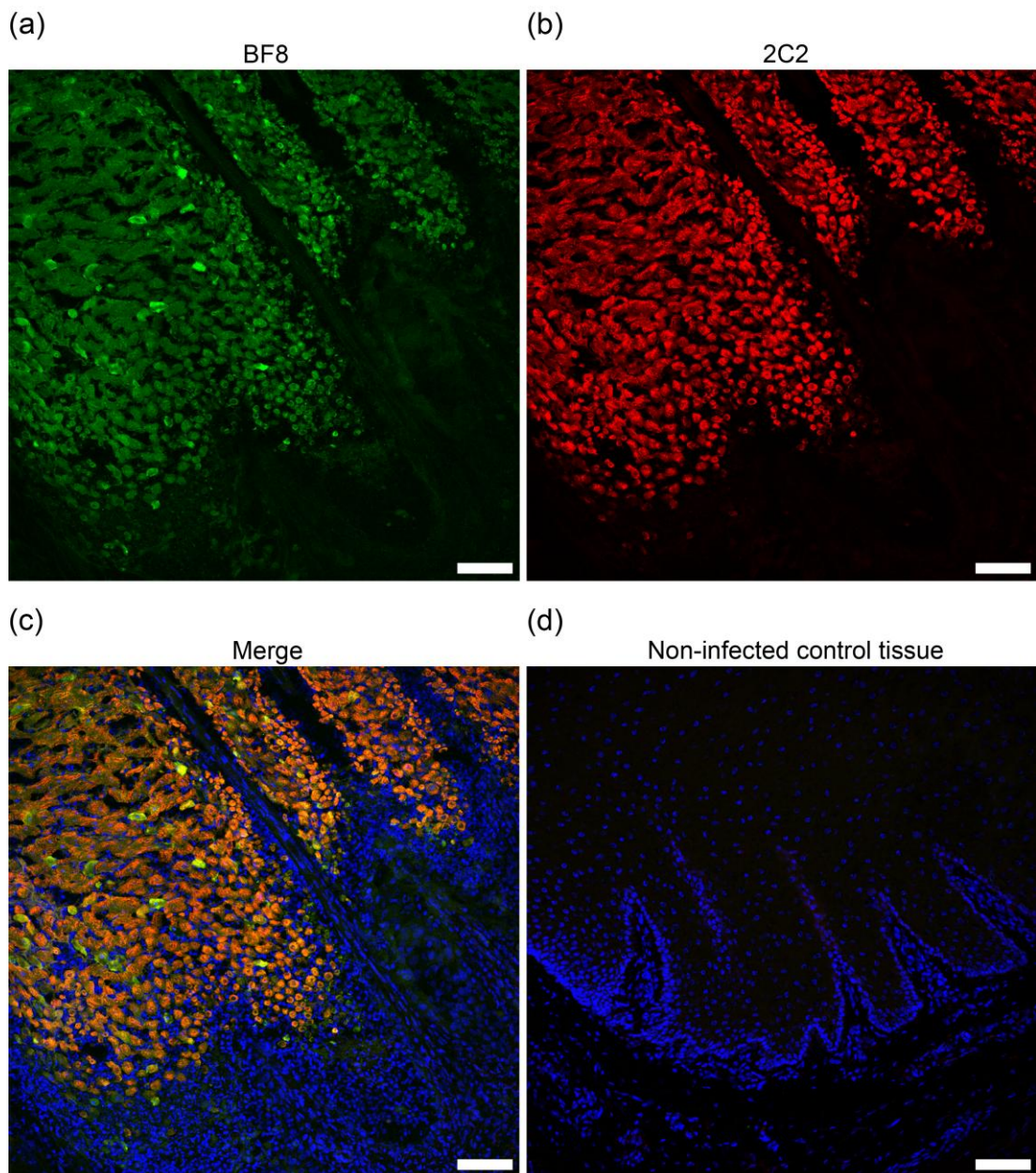


Figure 31. Infected and non-infected tongue epithelium stained with MAbs BF8 and 2C2. (a) to (c) Infected tongue epithelium cryosections harvested 4 days post-contact challenge. (a) FMDV capsids stained green (anti-FMDV capsid MAb BF8). (b) FMDV non-structural protein 3A stained red (anti-FMDV 3A MAb 2C2). (c) Merge image of (a) and (b) highlighting the co-localisation of FMDV capsid and 3A proteins. (d) No signal was detected with MAbs BF8 (green) or 2C2 (red) on non-infected control tissue. Nuclei stained blue (DAPI), scale bars represent 80µm.

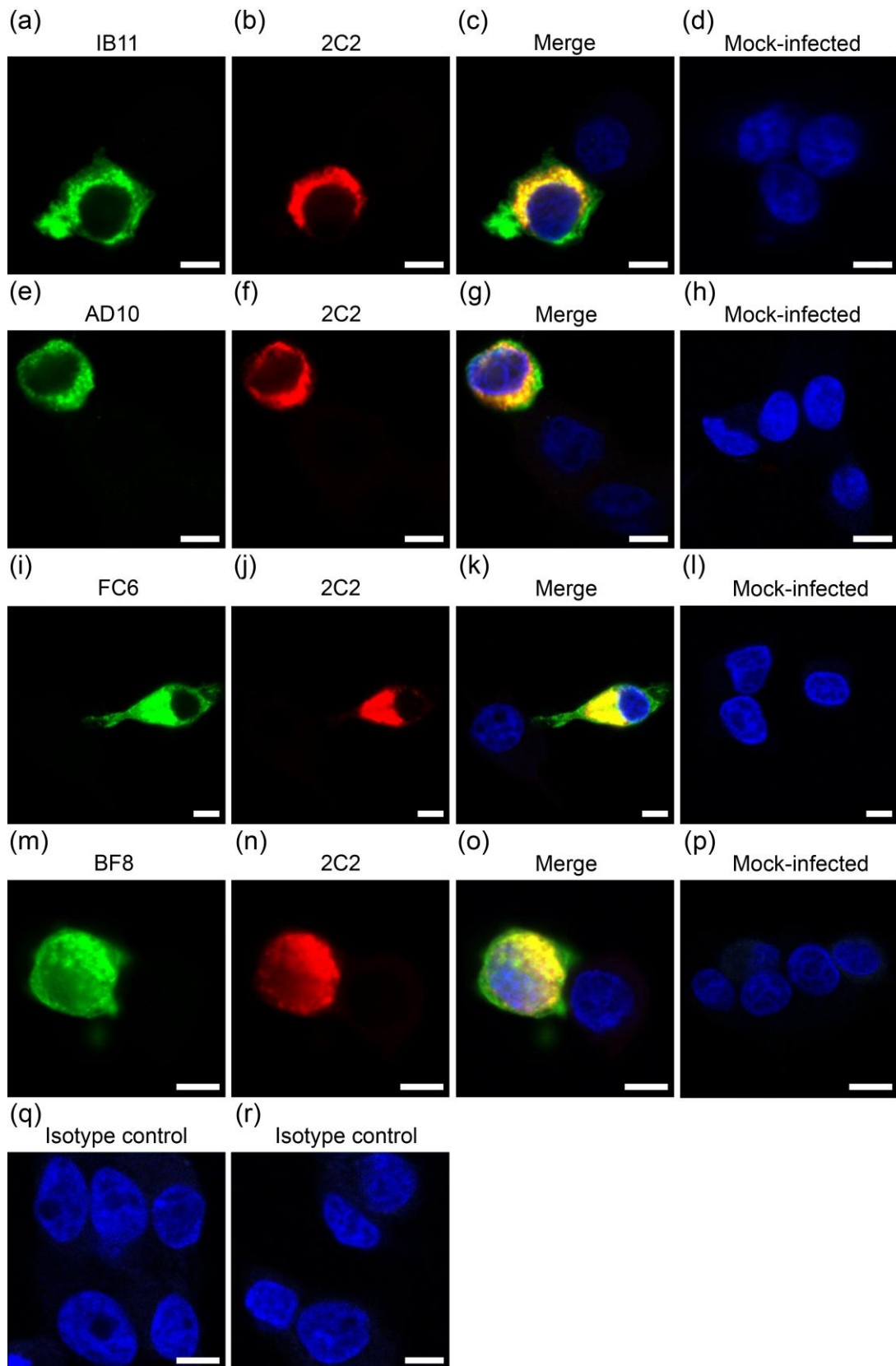


Figure 32. Anti-FMDV MAb validation on infected and mock-infected BHK-21 cells. Cells were fixed and labelled 5 hours after mock-infection (PBS) or FMDV O UKG 34/2001 infection at MOI 10. (a) to (c) FMDV capsid stained green (anti-FMDV

capsid MAb IB11), FMDV non-structural protein stained red (anti-FMDV 3A MAb 2C2). (d) No signal detected with MAbs IB11 (green) or 2C2 (red) on mock-infected cells. (e) to (g) FMDV capsid stained green (anti-FMDV capsid MAb AD10), FMDV non-structural protein stained red (anti-FMDV 3A MAb 2C2). (h) No signal detected with MAbs AD10 (green) or 2C2 (red) on mock-infected cells. (i) to (k) FMDV capsid stained green (anti-FMDV capsid MAb FC6), FMDV non-structural protein stained red (anti-FMDV 3A MAb 2C2). (l) No signal detected with MAbs FC6 (green) or 2C2 (red) on mock-infected cells. (m) to (o) FMDV capsid stained green (anti-FMDV capsid MAb BF8), FMDV non-structural protein stained red (anti-FMDV 3A MAb 2C2). (p) No signal detected with MAbs BF8 (green) or 2C2 (red) on mock-infected cells. (q) Merge image of FMDV infected cells stained with isotype control MAbs TRT3 (red, anti-turkey rhinotracheitis virus) and TRT1 (green, anti-turkey rhinotracheitis virus). (r) Merge image of FMDV infected cells stained with isotype control MAbs TRT3 (red) and AV29 (green, anti-chicken antigen). No signal was detected with the isotype control MAbs. Nuclei stained blue (DAPI). Scale bars represent 5 μ m.

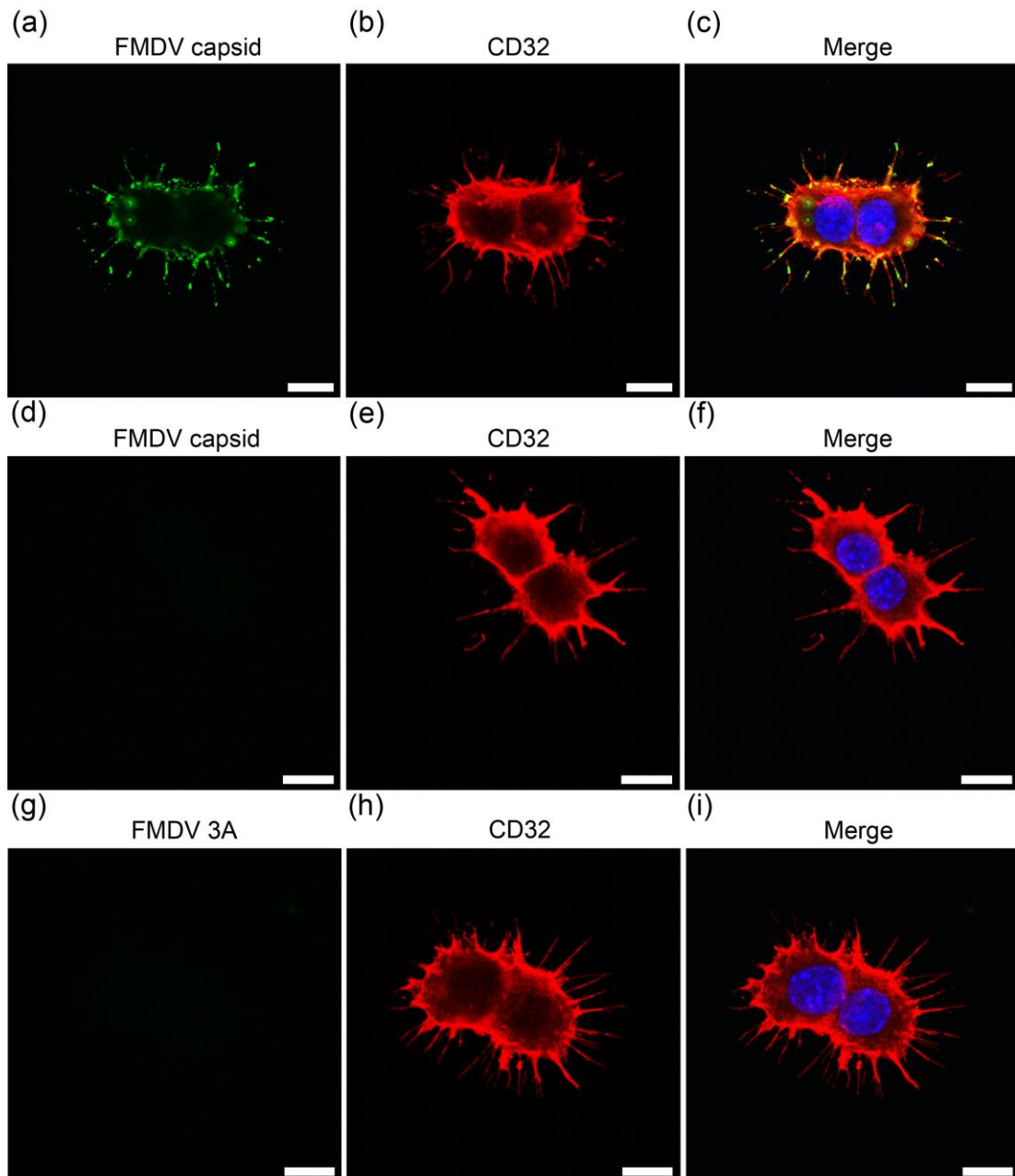


Figure 33. Detecting FMDV immune complexes *in vitro* on the surface of mouse fibroblast cells. (a) to (c) Mouse fibroblast 3T3 cells expressing bovine CD32 were paraformaldehyde fixed, washed and incubated with FMDV immune complexes prepared by incubating FMDV with heat inactivated cattle polyclonal immune serum. Cells were subsequently washed, fixed and stained. (a) FMDV capsid stained green (anti-FMDV capsid MAb IB11). (b) CD32 stained red (anti-CD32 MAb CCG37). (c) Merge image of (a) and (b), FMDV capsid stained green, CD32 stained red and nuclei stained blue (DAPI). (d) to (f) Cells prepared as described above except FMDV was incubated with non-immune cattle serum. (d) No FMDV capsid was detected (green, anti-FMDV capsid MAb IB11). (e) CD32 stained red (anti-CD32 MAb CCG37). (f) Merge image of (d) and (e), no FMDV capsid (green) detected, CD32 stained red and nuclei stained blue (DAPI). (g) to (i) Cells prepared as described above with FMDV immune complexes. (g) No FMDV non-structural protein 3A (green, anti-FMDV 3A MAb 2C2) was detected, consistent with lack of

FMDV replication and internalisation by fixed cells. (h) CD32 stained red (anti-CD32 MAb CCG36). (i) Merge image of (g) and (h), no FMDV non-structural protein 3A (green) detected, CD32 stained red and nuclei stained blue (DAPI). Scale bars represent 10µm.

2.4.4.3. Analysis of tissue samples collected from 1 to 4 days post-infection

The dorsal soft palates, pharyngeal tonsils, palatine tonsils, lateral retropharyngeal lymph nodes and mandibular lymph nodes were harvested from 8 cattle on days 1 to 4 post intradermolingual challenge and from a non-infected control animal.

Cryosections were screened with MAbs directed against FMDV capsid to determine the ability of the MAbs (Table 1) to detect FMDV in tissue not associated with vesicle formation. In addition, the sections were labelled with MAbs directed against 3A proteins, with consecutive sections labelled with isotype control MAbs (Table 1). No signal was detected with MAbs directed against FMDV on tissue from non-infected control animals, tissue harvested on day 1 post-infection ($n = 2$) or on dorsal soft palate, pharyngeal tonsil or lateral retropharyngeal lymph node sections.

FMDV capsid and 3A proteins were consistently detected in the palatine tonsil crypt epithelium from days 2 to 4 post-infection, a region of the palatine tonsil shown to express the integrin $\alpha v \beta 6$ ($n = 6$ animals. Figure 9 and Figure 34). FMDV 3A and capsid proteins co-localised in the cytoplasm of infected cells. A small number of infected cells were consistently detected in the cortex of mandibular lymph nodes with FMDV capsid and 3A MAbs, from days 2 to 4 post-infection ($n = 6$ animals, Figure 35). The phenotype of the cells was investigated by labelling cryosections with MAbs directed against FMDV in combination with MAbs specific for CD21, MHC class II, CD14, CD40 and the integrin $\alpha v \beta 6$ (Table 1). It was not possible to determine the phenotype of the infected cells on cryosections due to the expression of these markers by the encircling cells, as highlighted in Figure 36, with the infected

cell closely associated with a population of cells expressing CD21. The infected or encircling cells did not express the integrin $\alpha v \beta 6$ (Figure 35).

FMDV capsid was detected in the light zone of mandibular lymph node germinal centres as early as 3 to 4 days post intradermolingual challenge (n = 4 animals, Figure 37). No FMDV 3A was detected in association with the diffuse punctate pattern of labelled viral capsid.

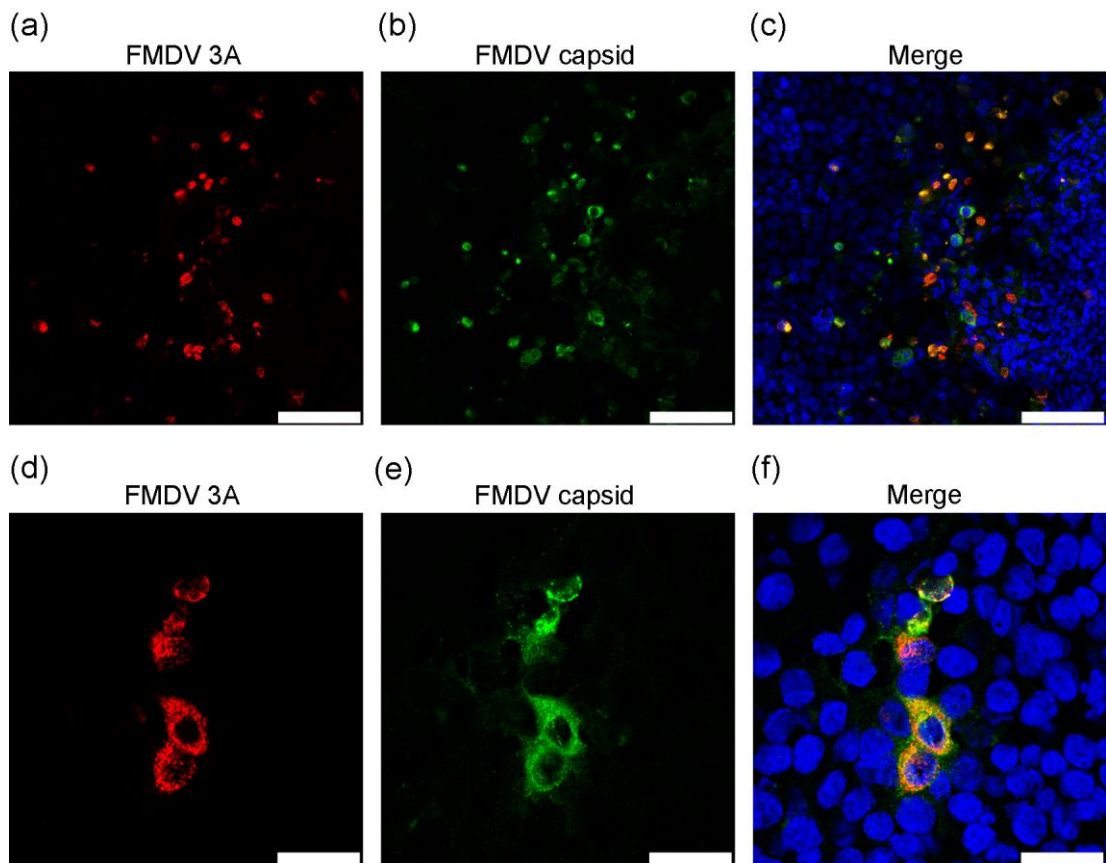


Figure 34. FMDV replicates in the palatine tonsil crypt epithelium.

(a) to (f) Palatine tonsil cryosections harvested 4 days post-intradermolingual challenge. (a) FMDV 3A protein (red, anti-FMDV 3A MAb 2C2) and (b) FMDV capsid protein (green, anti-FMDV capsid MAb IB11) were detected in the palatine tonsil crypt epithelium. (c) Merge image of (a) and (b). FMDV 3A stained red, FMDV capsid stained green, nuclei stained blue (DAPI). (d) to (f) Higher power images highlighting the cytoplasmic pattern and co-localisation of FMDV 3A (red, anti-FMDV 3A MAb 2C2) and FMDV capsid protein (green, anti-FMDV capsid MAb IB11). (f) Merge image of (d) and (e), FMDV 3A stained red, FMDV capsid stained green, nuclei stained blue (DAPI). Scale bars represent: (a) to (c), 50 μ m; (d) to (f), 20 μ m.

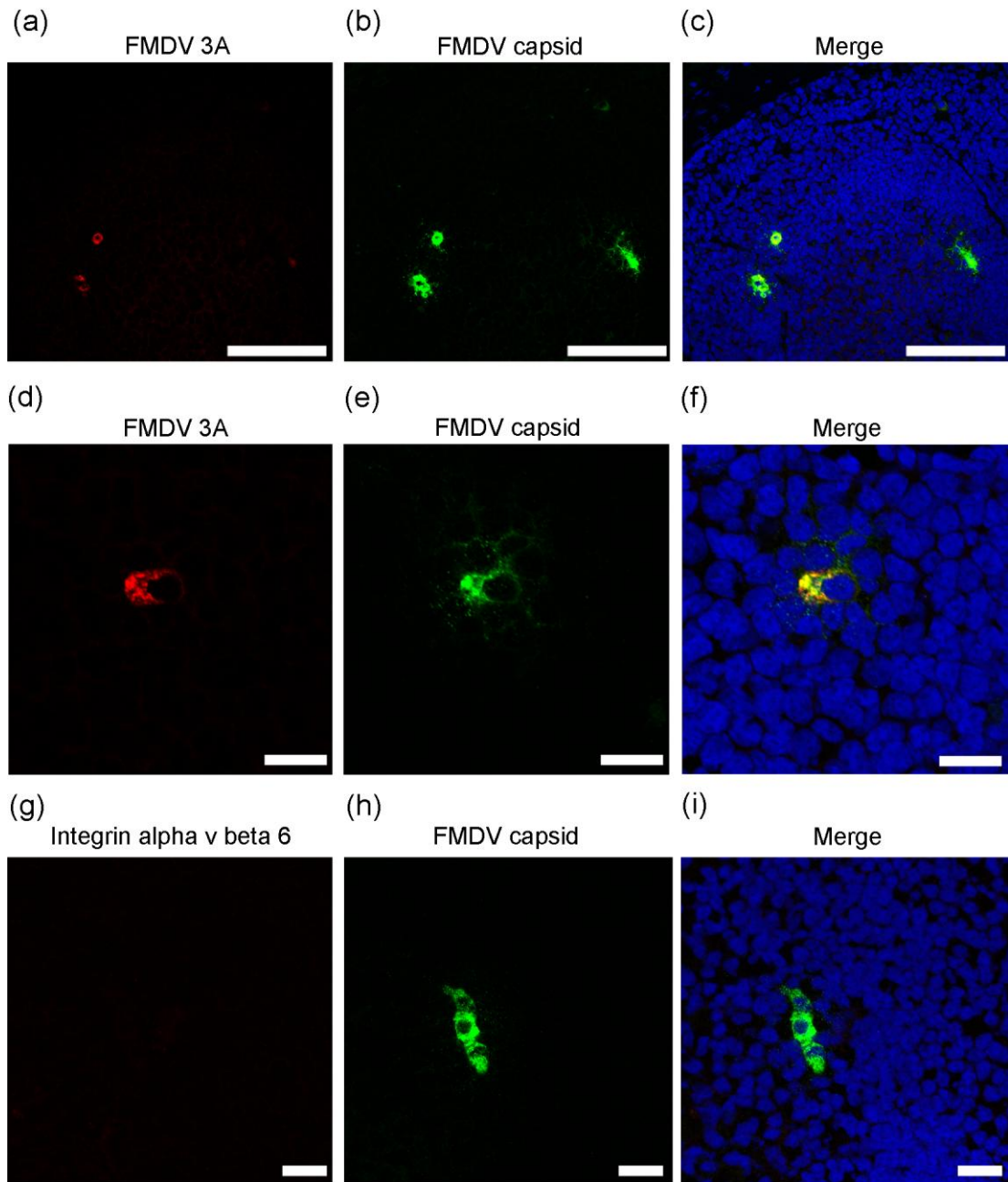


Figure 35. FMDV replicates in cells in the cortex of mandibular lymph nodes.

(a) to (i) Mandibular lymph node cryosections harvested 4 days post-intradermolingual challenge. (a) A small number of infected cells were detected in the lymph node cortex with MAb 2C2 (red, anti-FMDV 3A) and (b) MAb IB11 (green, anti-FMDV capsid). (c) Merge image of (a) and (b). FMDV 3A stained red, FMDV capsid stained green, nuclei stained blue (DAPI). (d) Higher power image of the mandibular lymph node cortex highlighting cytoplasmic FMDV 3A (red, anti-FMDV 3A MAb 2C2) and (e) capsid (green, anti-FMDV capsid MAb IB11). (f) Merge image of (d) and (e). FMDV 3A stained red, FMDV capsid stained green, nuclei stained blue (DAPI). Merge image highlights the cytoplasmic co-localisation of FMDV 3A and FMDV capsid in the mandibular lymph node cortex during the acute stages of infection. (g) No integrin $\alpha v \beta 6$ (red, anti- $\alpha v \beta 6$ MAb 10D5) was detected in the cortex of the mandibular lymph node. (h) FMDV capsid stained green

(anti-FMDV capsid MAb IB11). (i) Merge image of (g) and (h). No integrin $\alpha\text{v}\beta 6$ (red) was detected in association with FMDV capsid (green). Nuclei stained blue (DAPI), scale bars represent: (a) to (c), 100 μm : (d) to (i), 20 μm .

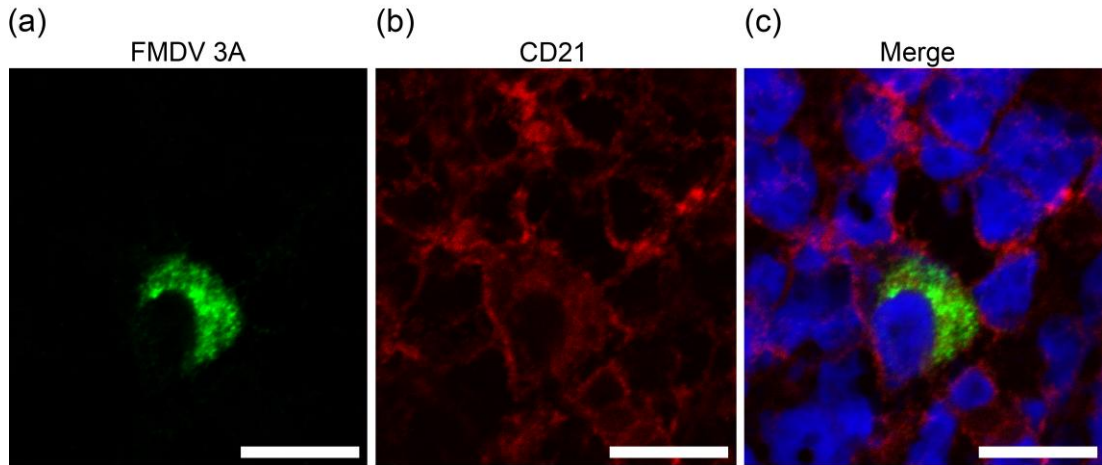


Figure 36. Cells supporting FMDV replication in mandibular lymph nodes were in close association with cells expressing CD21.

Mandibular lymph node cryosection harvested 4 days post-intradermolingual challenge. (a) FMDV 3A stained green (anti-FMDV 3A MAb 2C2). (b) CD21 expressing cells stained red (anti-CD21 MAb CC21). (c) Merge image of (a) and (b). FMDV 3A stained green, CD21 stained red. Nuclei stained blue (DAPI), scale bars represent 10 μm .

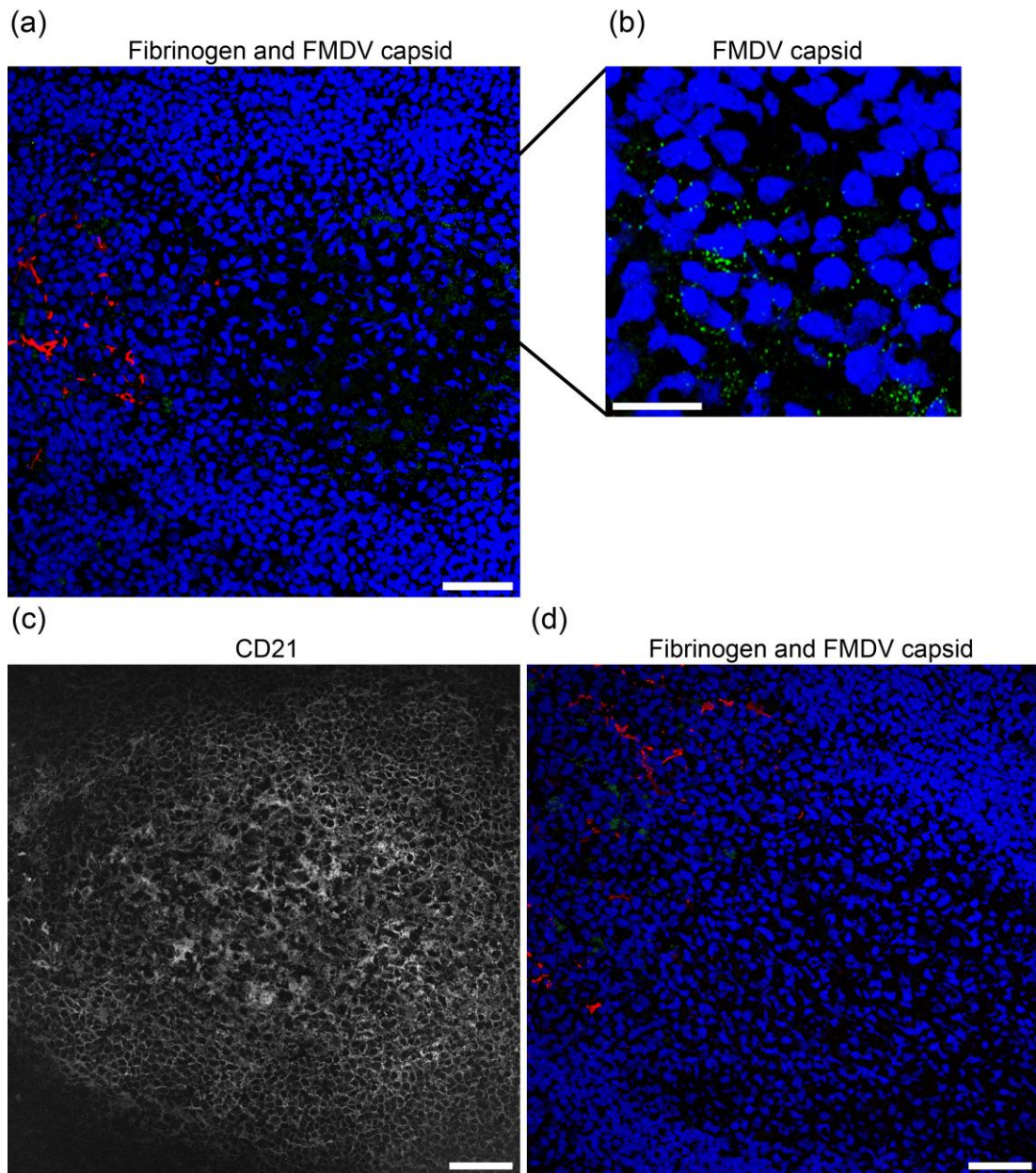


Figure 37. FMDV capsid detected in the light zone of mandibular lymph node germinal centres harvested 4 days post-intradermolingual challenge.

(a) to (c) Mandibular lymph node cryosection harvested 4 days post-intradermolingual challenge. (a) Fibrinogen, associated with dark zone FDCs, stained red (anti-fibrinogen MAb D46). FMDV capsid stained green (anti-FMDV capsid MAb IB11). (b) Higher power image of the diffuse punctate pattern of viral capsid (green, anti-FMDV capsid MAb IB11) associated with cells in the germinal centre light zone. (c) CD21 stained gray (anti-CD21 MAb CC51). (d) Mandibular lymph node cryosection harvested from a non-infected control animal. Fibrinogen stained red (anti-fibrinogen MAb D46). No signal was detected with MAb IB11 (green, anti-FMDV capsid MAb). Nuclei stained blue (DAPI), scale bars represent: (a), (c) and (d), 100µm; (b), 25µm.

2.4.4.4. Analysis of tissue samples collected from 29 to 38 days post-contact infection

To determine whether viral RNA detected by LCM and *in situ* hybridization was associated with viral structural and non-structural proteins; cryosections from the dorsal soft palates, pharyngeal tonsils, palatine tonsils, lateral retropharyngeal lymph nodes and mandibular lymph nodes collected from 29 to 38 days post-contact infection were analysed with MAbs directed against FMDV capsid, 3A and 3C proteins (Table 1).

The anti-FMDV capsid MAbs gave a diffuse punctate pattern of positive labelling which was restricted to germinal centres within lymphoid tissue and confined to the light zone within the germinal centre from 29 days post-infection (Table 5, Figure 38, Figure 39). In contrast, the FMDV non-structural proteins 3A and 3C could not be detected in any of the tissue from animals after 28 days post-contact infection. The diffuse punctate pattern of labelled viral capsid was shown to be localised to the light zone FDC network by co-labelling with an antibody specific for light zone FDCs (Figure 40). Analysis of *in situ* hybridization and immunohistochemistry showed a consistent punctate pattern (Figure 41). The punctate labelling pattern observed in Figure 41 is consistent with the distribution pattern of iccosomes on FDCs (Szakal *et al.*, 1988). This pattern is in contrast to the diffuse cytoplasmic labelling pattern of cells observed during acute infection *in vivo* and in infected cells *in vitro* (sections 2.4.4.1 and 2.4.4.3).

Table 5. Immunohistochemical analysis of tissue 29 to 38 days post-contact infection for FMDV capsid and non-structural proteins.

Tissue	Number of animals sampled	FMDV capsid +ve GCs*
DSP	17	0
Pharyngeal tonsils	10	0
Palatine tonsils	10	6
RPLN	10	8
MLN	22	22

Tissue was negative by immunohistochemical analysis for FMDV non-structural proteins.

* Number of animals with germinal centres (GCs) positive for FMDV capsid.

DSP = dorsal soft palate.

RPLN = lateral retropharyngeal lymph node.

MLN = mandibular lymph node.

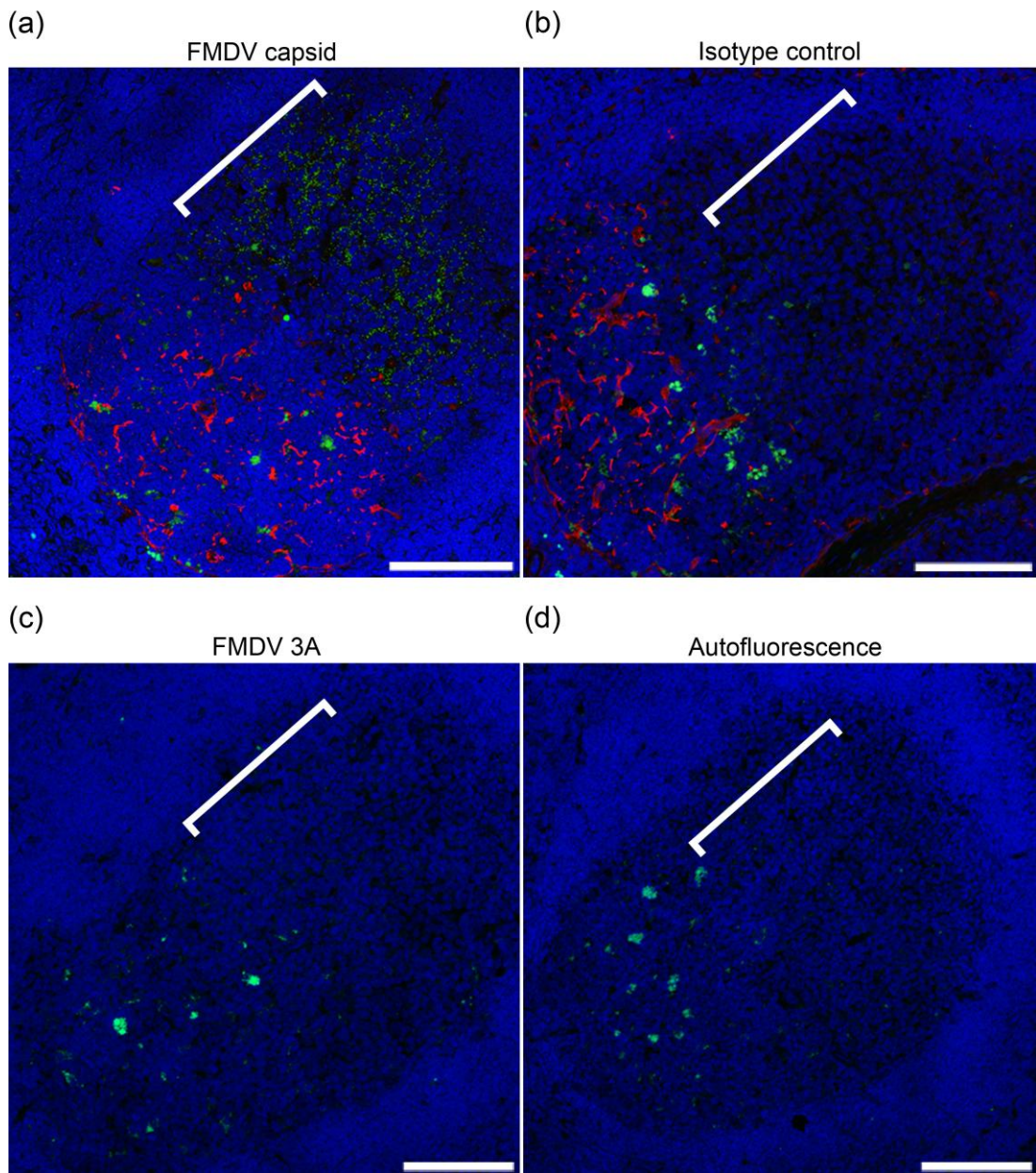


Figure 38. FMDV capsid was restricted to lymphoid tissue germinal centres from 29 days post-infection.

(a) to (d) Mandibular lymph node germinal centre sections harvested 38 days post-contact infection, the white markers demarcate the germinal centre light zones. (a) FMDV capsid stained green (anti-FMDV capsid MAb IB11), dark zone FDCs stained red (anti-fibrinogen MAb D46). FMDV capsid is restricted to the germinal centre light zone. (b) Dark zone FDCs stained red (anti-fibrinogen MAb D46). No specific signal detected in the germinal centre light zone with isotype primary control MAb TRT1 (green, anti-turkey rhinotracheitis virus). A higher power image of (a) and (b) is displayed in Figure 39. (c) No signal detected in the germinal centre light zone with FMDV non-structural protein 3A (green, anti-FMDV 3A MAb 2C2). FMDV non-structural proteins could not be detected by immunohistochemical analysis of tissue from 29 to 38 days post-contact infection. (d) No primary or secondary antibodies highlighting autofluorescence associated with bovine germinal

centres. The majority of the autofluorescent signal is restricted to the germinal centre dark zone. Nuclei stained blue (DAPI), scale bars represent 100µm.

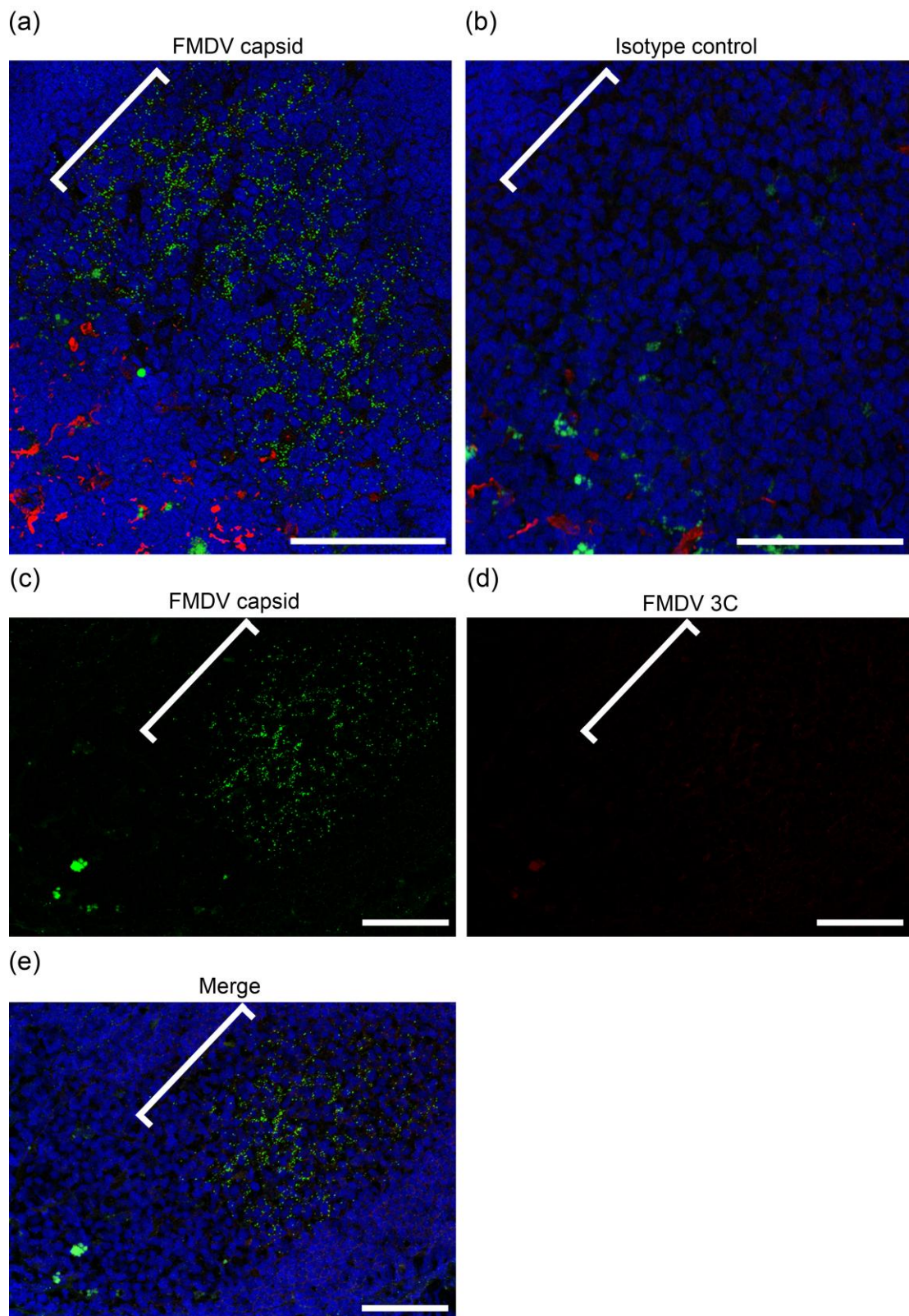


Figure 39. FMDV capsid detected in mandibular lymph node germinal centres.

(a) and (b) Mandibular lymph node germinal centre sections harvested 38 days post-contact infection, the white markers demarcate the germinal centre light zones. (a) FMDV capsid labelled green (anti-FMDV capsid MAb IB11), dark zone FDCs labelled red (anti-fibrinogen MAb D46). FMDV capsid is restricted to the germinal

centre light zone. (b) Dark zone FDCs labelled red (anti-fibrinogen MAb D46), no specific signal detected in the germinal centre light zone with isotype primary control MAb TRT1 (green, anti-turkey rhinotracheitis virus). (c) to (e) Mandibular lymph node germinal centre section harvested 38 days post-contact infection, the white markers demarcate the germinal centre light zone. (c) FMDV capsid stained green (anti-FMDV capsid MAb IB11). (d) No FMDV 3C protein detected in the germinal centre light zone (red, anti-FMDV 3C MAb 3C1). (e) Merge image of (c) and (d). Nuclei stained blue (DAPI). FMDV capsid (green) is restricted to the germinal centre light zone. The majority of the autofluorescent signal is restricted to the germinal centre dark zone. Scale bars represent 100µm.

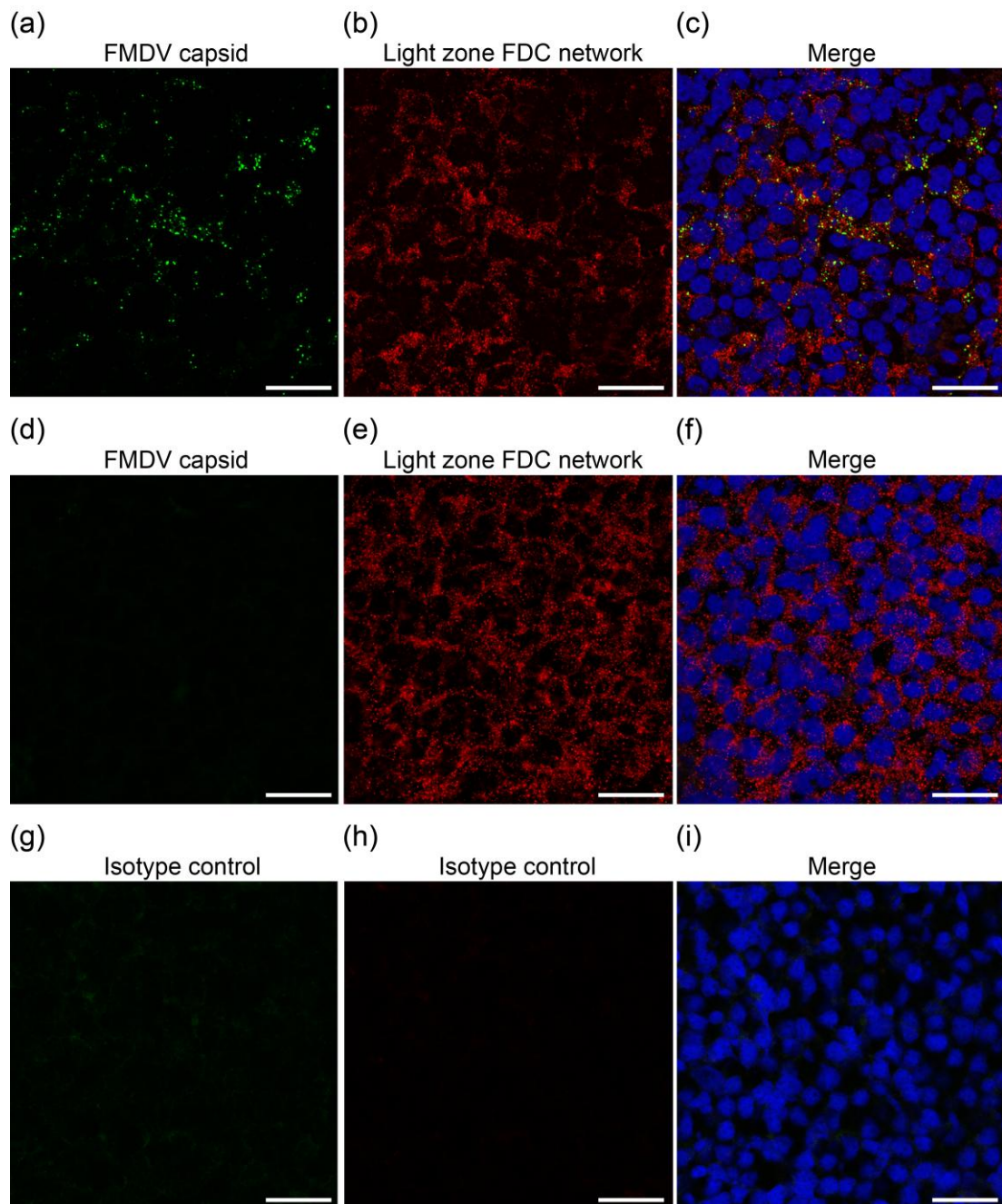


Figure 40. The diffuse punctate pattern of viral capsid was shown to be localised to the light zone FDC network by co-staining with an antibody specific for light zone FDCs.

(a) to (c) A mandibular lymph node cryosection harvested 38 days post-contact infection. (a) FMDV capsid stained green (anti-FMDV capsid MAb IB11). (b) Light zone FDC network stained red (anti-light zone FDC MAb CNA.42). (c) Merge image of (a) and (b) highlighting the diffuse punctate pattern associated with FMDV capsid (green) linked to the light zone FDC network (red). Nuclei stained blue (DAPI). (d) to (f) Mandibular lymph node cryosection harvested from a non-infected control animal. (d) No signal detected using MAb IB11 (green, anti-FMDV capsid). (e) Light zone FDC network stained red (anti-light zone FDC MAb CNA.42). (f) Merge image of (d) and (e). No FMDV capsid (green) detected, light zone FDC network stained red, nuclei stained blue (DAPI). (g) to (i) A mandibular lymph node cryosection harvested 38 days post-contact infection. (g) No signal detected with

isotype matched control MAb TRT1 (green, anti-turkey rhinotracheitis virus). (h) No signal detected with isotype matched control MAb AV48 (red, anti-chicken antigen). (i) Merge image of (g) and (h). Nuclei stained blue (DAPI). Scale bars represent 20µm.

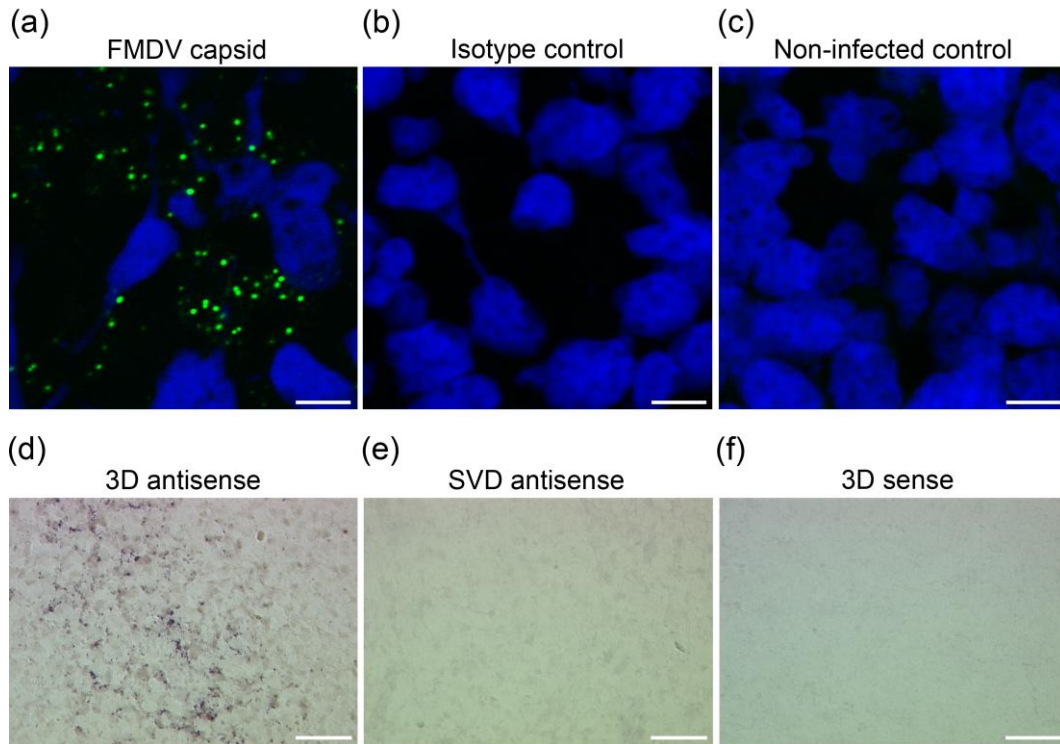


Figure 41. High power images comparing the pattern of FMDV detected 38 days post-contact infection by immunohistochemical analysis and by *in situ* hybridization.

(a) and (b) Mandibular lymph node cryosections harvested 38 days post-contact infection. (a) FMDV capsid stained green (anti-FMDV capsid MAb IB11), nuclei stained blue (DAPI). (b) No signal detected with isotype matched control MAb TRT1 (green, anti-turkey rhinotracheitis virus), nuclei stained blue (DAPI). (c) Mandibular lymph node cryosection harvested from a non-infected control animal. No signal detected with MAb IB11 (green, anti-FMDV capsid), nuclei stained blue (DAPI). Scale bars represent 5µm. (d) to (f) Mandibular lymph node cryosections harvested 38 days post-contact infection and analysed by *in situ* hybridization with (d) FMDV 3D antisense RNA probe, (e) swine vesicular disease (SVD) antisense RNA control probe and (f) FMDV 3D sense RNA control probe. No counterstain, scale bars represent 50µm. Panels (a) and (d) highlight the similar diffuse punctate staining pattern using *in situ* hybridization to detect FMDV 3D RNA and MAb IB11 to detect FMDV capsids.

2.4.5. Virus isolation

2.4.5.1. Evaluation of CD32 expressing cells used for virus isolation

The ability of BHK-21 cells or BHK-21 cells expressing either bovine CD32 or bovine CD32tail– mutant (Peltz *et al.*, 1988) to bind and phagocytose IgG-coated particles was evaluated by uptake studies of immune complexed FITC-ovalbumin (Figure 42). BHK-21 cells expressing CD32 were able to bind and phagocytose immune complexed FITC-ovalbumin. BHK-21 cells expressing CD32tail– mutant were able to bind immune complexed FITC-ovalbumin but ingestion of IgG coated particles was inefficient, which is consistent with published data for isoforms of CD32 lacking the cytoplasmic domain (Tuijnman *et al.*, 1992). Non-transfected BHK-21 cells did not bind or internalise immune complexed FITC-ovalbumin.

The virus neutralisation test was used to compare the ability of serum from 4 animals 13 days or more post-infection, to neutralise virus in the presence of BHK-21 cells and BHK-21 cells expressing CD32. An example of an assay is displayed in Figure 43. The serum was consistently less efficient, by one or two doubling dilutions, at neutralising virus in the presence of BHK-21 cells expressing CD32, suggesting that these cells were more susceptible to virus in the presence of specific antibody compared to standard BHK-21 cells.

Monolayers of MΦ (kindly provided by L Robinson who also kindly helped with the analysis of these experiments) and BHK-21 cells expressing CD32 were spiked with homogenised palatine tonsil and mandibular lymph node supernatants from a control

animal. The cells were subsequently exposed to FMDV or FMDV immune complexes for 6 hours and analysed by flow cytometry for viral non-structural proteins (Figure 44). Immune complexed FMDV was readily detectable in MΦ by flow cytometry at MOI 1 in the presence of homogenised lymph node supernatants. BHK-21 cells expressing CD32 were more susceptible to virus in the presence of specific antibody as shown by the virus neutralisation test. However, detection of immune complexes in these cells by flow cytometry in the presence of lymphoid tissue homogenates was not sufficiently sensitive due to a high degree of background staining detected with isotype control MAbs. Therefore, only MΦ were used for the detection of FMDV in lymphoid tissue by flow cytometry.

2.4.5.2. Virus isolation from tissue samples collected 29 to 38 days post-contact infection

The palatine tonsils, lateral retropharyngeal lymph nodes and mandibular lymph nodes of 8 animals were harvested between 29 and 38 days post-contact infection for processing in preparation for virus isolation as described under section 2.3.21. No FMDV 3A was detected in CD32 expressing cell lines and no virus was isolated on BTY cells. An example of a negative flow cytometry data set for the detection of FMDV 3A in a tissue homogenate of a mandibular lymph node harvested 29 days post-contact infection and inoculated onto MΦ, is displayed in Figure 45.

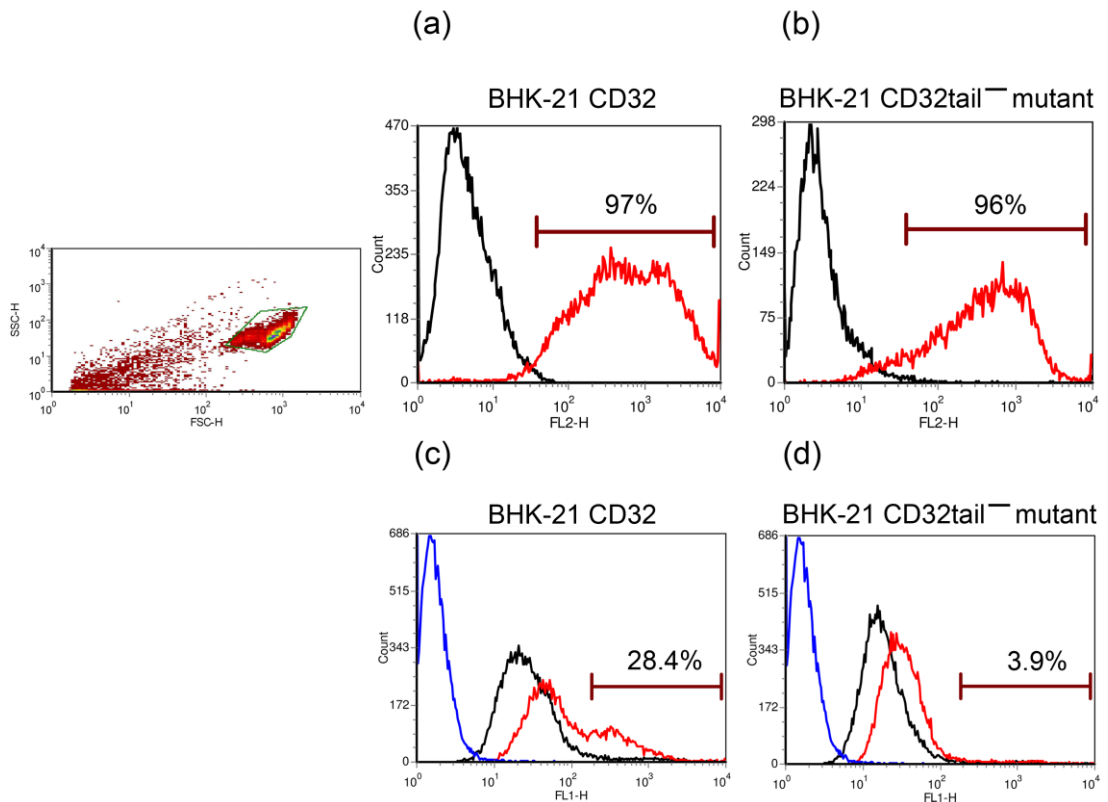


Figure 42. Binding and phagocytosis studies of BHK-21 cells or BHK-21 cells expressing CD32 and CD32tail⁻ mutant.

(a) and (b) The percentages of viable BHK-21 cells used for subsequent phagocytosis studies expressing CD32 (BHK-21 CD32) or CD32tail⁻ mutant (BHK-21 CD32tail⁻ mutant) were evaluated by flow cytometry. Cells were labelled with anti-CD32 MAb CCG36 (red line) or isotype control MAb TRT1 (black line). The markers represent the percentages of gated cells labelled with MAb CCG36. (c) and (d) The ability of BHK-21 cells or BHK-21 cells expressing either (c) bovine CD32 or (d) bovine CD32tail⁻ mutant to bind and phagocytose IgG-coated particles was evaluated by uptake studies of immune complexed FITC-ovalbumin. (c) and (d) BHK-21 cells did not bind or phagocytose immune complexed FITC-ovalbumin after incubation at 37°C for 30 minutes (blue lines). (c) BHK-21 cells expressing CD32 were able to bind immune complexed FITC-ovalbumin at 4°C (black line) and phagocytose immune complexed FITC-ovalbumin at 37°C (red line, 28.4%). (d) BHK-21 cells expressing CD32tail⁻ mutant were able to bind immune complexed FITC-ovalbumin at 4°C (black line) but ingestion of IgG coated particles at 37°C (red line, 3.9%) was inefficient.

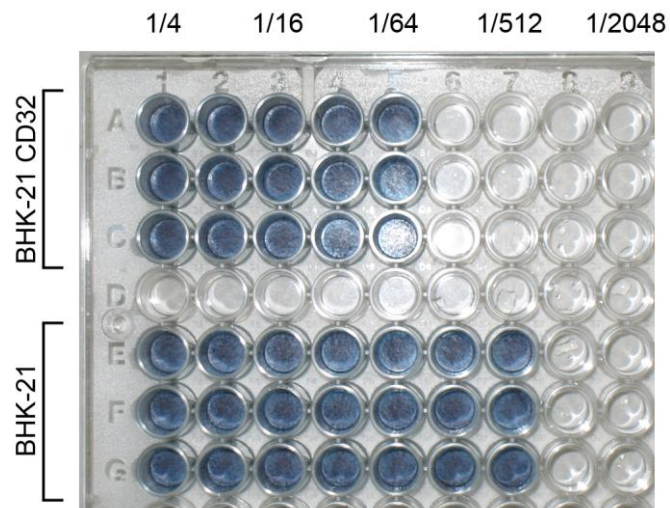


Figure 43. A comparison of the ability of serum to neutralise a fixed dose of virus in the presence of BHK-21 cells and BHK-21 cells expressing CD32.

An example of a virus neutralisation test used to compare the ability of serum from an animal 13 days post-infection, to neutralise virus in the presence of BHK-21 cells and BHK-21 cells expressing CD32. The serum was consistently less efficient at neutralising virus in the presence of BHK-21 cells expressing CD32, suggesting that these cells were more susceptible to virus in the presence of specific antibody compared to standard BHK-21 cells.

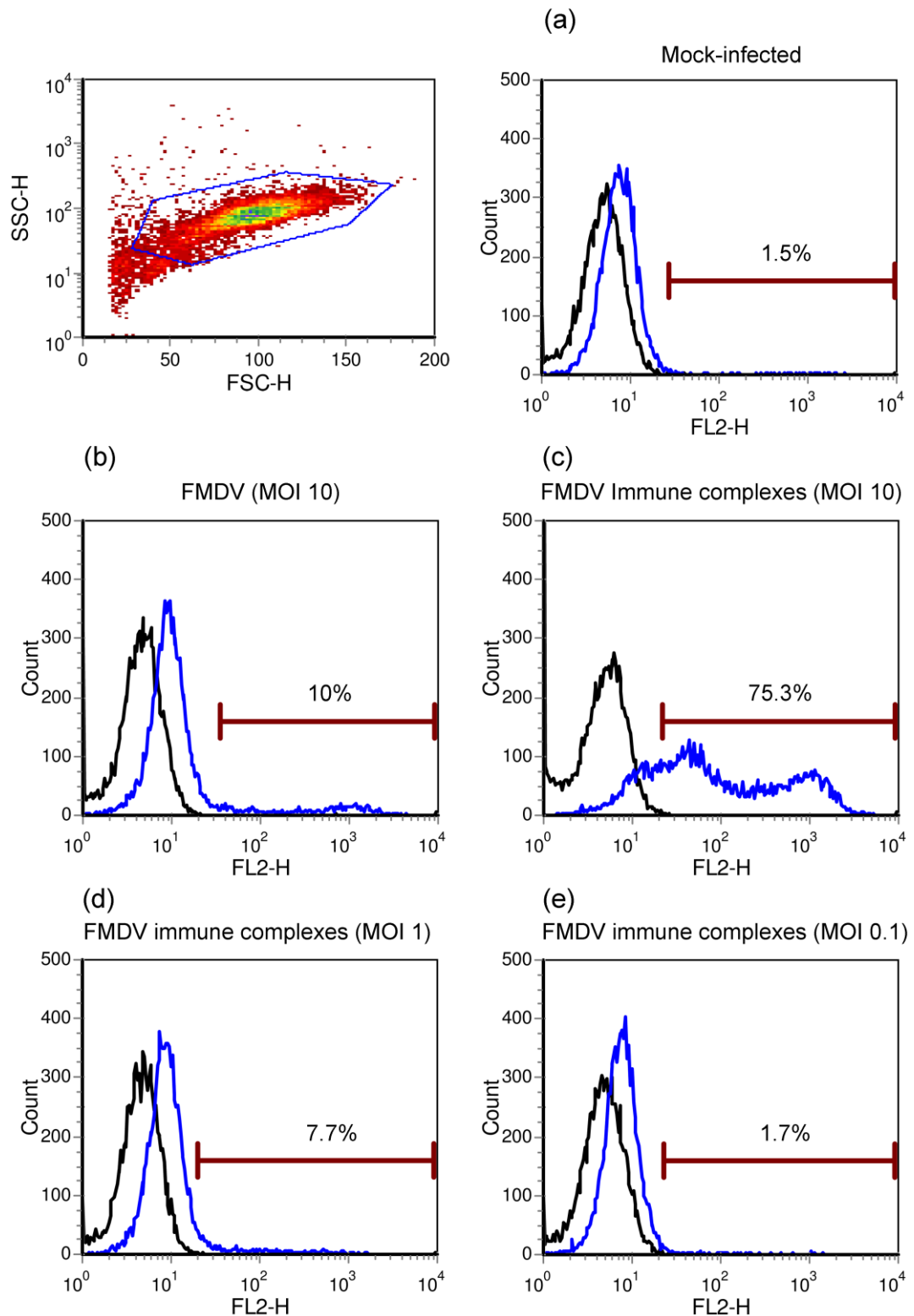


Figure 44. MΦ spiked with homogenised lymph node supernatant and exposed to FMDV and FMDV immune complexes.

Monolayers of MΦ in 6 well plates were spiked with homogenised mandibular lymph node supernatants from a control animal and either (a) mock-infected, (b) exposed to FMDV at MOI 10 or (c) to (e), exposed to FMDV immune complexes formed with immune serum at MOI 10 to MOI 0.1. Cells were exposed for 6 hours, labelled with anti-FMDV 3A MAb 2C2 (blue line) or isotype control MAb TRT3

(black line). The markers represent the percentages of gated cells labelled with MAb 2C2. Immune complexed FMDV was detectable at MOI 1 in the presence of homogenised lymph node supernatants.

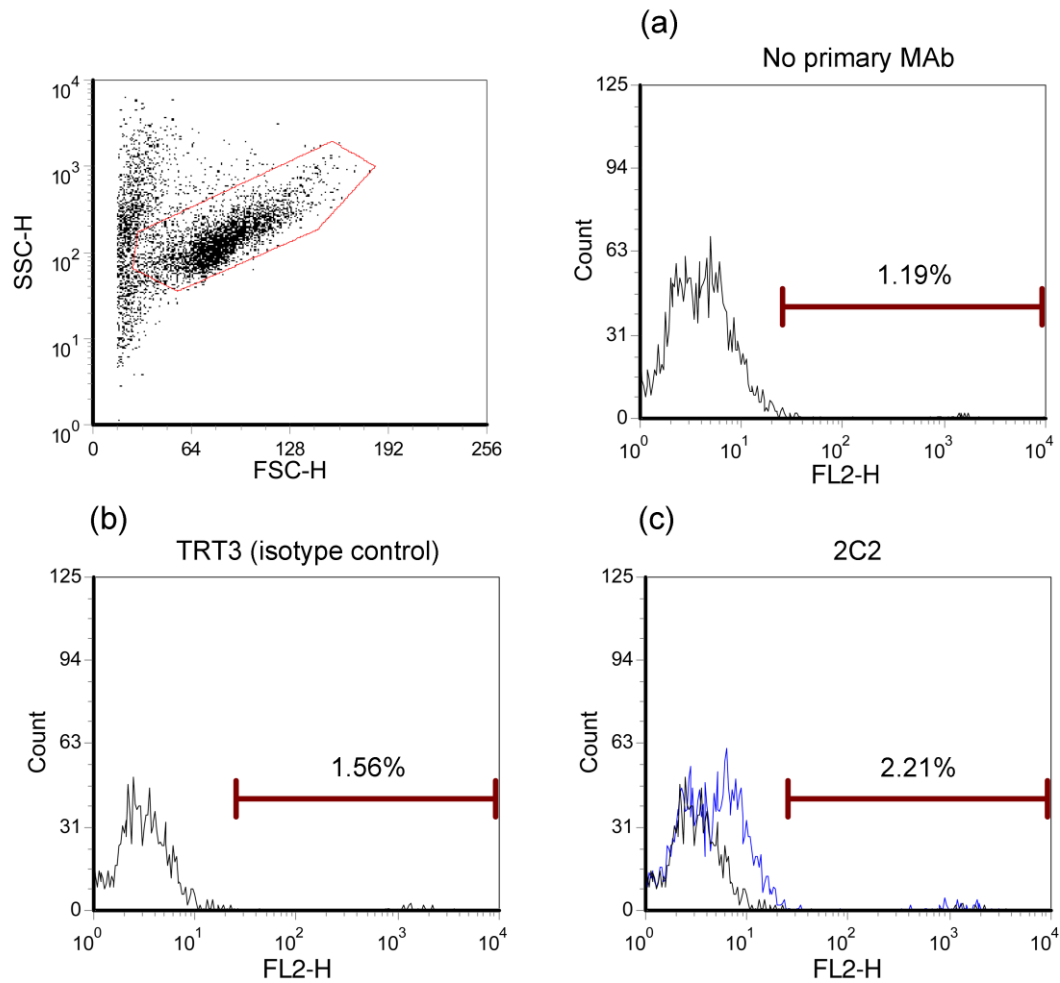


Figure 45. Flow cytometry analysis of MΦ inoculated with mandibular lymph node homogenate harvested 29 days post-contact infection.

Monolayers of MΦ in 6 well plates were inoculated with 100μL of mandibular lymph node homogenate harvested 29 days post-contact infection. Cells were exposed for 6 hours, followed by flow cytometry to detect FMDV 3A. (a) Cells labelled with secondary MAb only (1.19%). (b) Cells labelled with isotype control Mab (1.56%). (c) Cells labelled with isotype control MAb (black line) and anti-FMDV 3A MAb 2C2 (blue line). The marker represents the percentage of gated cells labelled with MAb 2C2 (2.21%). No virus was detected in MΦ scrapings by subsequent virus isolation on BTY cells.

2.5. Discussion

We have shown that FMDV genome, using LCM and quantitative rRT-PCR, can be detected consistently in germinal centres within the dorsal soft palate, pharyngeal tonsil, palatine tonsil, lateral retropharyngeal lymph node and mandibular lymph node at 38 days post-contact infection. Also, FMDV genome in these tissues was restricted to the germinal centre. These findings were confirmed with *in situ* hybridization studies, which revealed FMDV 3D RNA in germinal centres of lymphoid tissue but not in other compartments of these tissues. Using MAbs specific for conformational, non-neutralising epitopes of the FMDV capsid, we identified viral structural proteins restricted to the light zone FDC network of germinal centres within mandibular lymph nodes, lateral retropharyngeal lymph nodes and palatine tonsils up to 38 days post-contact infection, but not in the dorsal soft palates or pharyngeal tonsils. The inability to detect FMDV capsid in the dorsal soft palates and pharyngeal tonsils by immunohistochemistry is in contrast to the clear detection of FMDV genome by LCM. This inconsistency may be a consequence of differences in assay sensitivity or genomic RNA persisting longer than virus (Simon *et al.*, 2007). The diffuse punctate pattern of labelled viral capsid in tissue from 29 to 38 days post-infection, similar to the FMDV genome staining pattern detected by *in situ* hybridization, was in contrast to the diffuse cytoplasmic pattern observed in cells during acute infection *in vivo* and in infected cells *in vitro*.

The mandibular lymph nodes had notably more germinal centres containing FMDV capsid compared to the lateral retropharyngeal lymph nodes and palatine tonsils. This is consistent with the detection of significantly more FMDV genome copies per 10^8

copies of 28s rRNA in replicates of six germinal centres from mandibular lymph nodes, compared to similar replicates harvested from other tissues. FMDV capsid was detected in mandibular lymph node germinal centres of all animals examined between 29 to 38 days post-contact infection (n = 22), including five animals where FMDV could not be recovered by virus isolation or detected by rRT-PCR analysis of oropharyngeal scrapings collected at post-mortem 29 to 34 days post-infection using probang sampling cups (Alexandersen *et al.*, 2002). These results indicate that virus is likely to persist in all cattle to some degree following infection. This predilection to the mandibular lymph node is not surprising because afferent lymphatics of the mandibular lymph nodes in cattle drain the oral cavity and tongue, which are important sites of viral replication during the acute phase of infection. However, these results do not support findings from previous studies which reported detection of viral RNA by *in situ* hybridization and whole tissue quantitative rRT-PCR in the dorsal soft palate epithelium in 'carrier' animals (Prato Murphy *et al.*, 1999, Zhang and Alexandersen, 2004, Zhang and Kitching, 2001). We did not detect viral RNA in the epithelial compartments of all the tissues examined either by LCM and quantitative rRT-PCR or *in situ* hybridization, although we routinely detected viral RNA and capsid in germinal centres of these tissues.

Although MAbs specific for FMDV non-structural proteins could detect infected cells *in vitro* and *in vivo* during the acute phase of infection, no FMDV non-structural proteins were detected in any of the tissues examined from 29 days post-contact infection. The absence of detectable FMDV non-structural proteins indicates that the presence of viral RNA is not associated with active viral replication (Brocchi *et al.*,

1998, De Diego *et al.*, 1997). The finding of close co-localisation of viral RNA and capsid conformational epitopes, in the absence of non-structural proteins, supports the hypothesis that FMD viral particles or immune complexes are maintained in germinal centre light zones in a non-replicating state.

Interestingly, FMDV capsid was detected in the light zone of mandibular lymph node germinal centres as early as 3 to 4 days post intradermolingual challenge (n = 4).

FMDV is known to use members of the integrin family to initiate infection (Monaghan *et al.*, 2005). Current evidence from *in vitro* and *in vivo* studies indicates that $\alpha\beta6$ integrin serves as the major cellular receptor for FMDV. Since the distribution of $\alpha\beta6$ expression in cattle, namely in epithelial cells in the tongue, interdigital skin and coronary band (Monaghan *et al.*, 2005) correlates closely with the sites of FMDV replication, it is thought to determine the tissue tropism of the virus. We have shown by immunofluorescence confocal microscopy that $\alpha\beta6$ is not expressed in germinal centres, indicating that the early localisation of FMDV to germinal centre light zones is independent of $\alpha\beta6$ expression. Binding of virus to light zone germinal centre cells during the early stages of infection may play an important role in facilitating a FMDV B-cell response (Allen and Cyster, 2008, Gatto *et al.*, 2007, Kikuno *et al.*, 2007).

The results of these studies have important implications for understanding both the mechanism of viral persistence and the ability of FMDV infection to stimulate long-lasting antibody responses. FDCs are known to be non-endocytic cells capable of capturing and retaining antigen in the form of immune complexes for long periods of

time (Haberman and Shlomchik, 2003, Mandel *et al.*, 1980). Retention of immune complexed FMDV particles within lymphoid tissue represents a possible source of the infectious material detected by pharyngeal sampling of infected cattle either by direct harvesting of mucosal associated lymphoid tissue germinal centres or sampling of secondary cells, for example macrophages, DCs or B cells, able to support a low level virus replication cycle in the presence of high titres of neutralising antibodies (Mason *et al.*, 1993, Rigden *et al.*, 2002, Robinson, 2008). Of the tissue examined in the present study, only material from the palatine tonsils and pharyngeal tonsils are likely to be represented in probang samples. Viral RNA was detected in germinal centres of palatine tonsils and pharyngeal tonsils but capsid antigen was only detected in germinal centres of palatine tonsils making this tissue a likely source of infectious virus detected by probang sampling in cattle. However, it must be stressed that there are other areas of lymphatic tissue represented in probang samples which were not examined during the present study, for example, the lingual tonsils which have been shown to contain FDCs (Rebmann and Gasse, 2008).

FDCs are notoriously difficult cells to isolate and work with, infectious FMDV could not be isolated from the lymphoid tissue during these studies, most likely due to technical difficulties extracting virus from the tissue and working with the bovine system. Retention of other viruses such as HIV in a replication-competent state within the light zone of germinal centres has been reported and the next step will require the development and interrogation of murine model systems (Smith *et al.*, 2001). The previous observation that dexamethasone treatment suppresses the ability to detect FMDV in oropharyngeal scrapings (Ilott *et al.*, 1997) is consistent with the

hypothesis that the germinal centre is the reservoir for infectious virus, since glucocorticoid administration to mice is known to result in atrophy of the FDC network (Murray *et al.*, 2004). The recrudescence of virus in pharyngeal scrapings after dexamethasone treatment could be a consequence of the failure of the treatment to completely eliminate structures capable of maintaining viable virus. FDC-trapped HIV has been shown to represent a significant reservoir of infectious and highly diverse HIV, demonstrating greater genetic diversity than most other tissues, providing drug-resistant and immune-escape quasispecies that contribute to virus transmission, persistence and diversification (Keele *et al.*, 2008). Retention of intact FMDV particles on the FDC network would therefore provide an ideal mechanism of maintaining a highly cytopathic and lytic virus like FMDV extracellularly in a non-replicating, native, stable non-degraded state (Smith *et al.*, 2001, Tew and Mandel, 1979). This reservoir could serve as the source of genetically diverse viral mutants (quasispecies), detected in 'carrier' animals (Domingo *et al.*, 2002, Vosloo *et al.*, 1996), able to infect susceptible cells that come into contact with the FDC network.

FMDV infection in ruminants elicits an immune response that can provide protection for several years (Cunliffe, 1964) and the degree of protection correlates well with specific SNTs (Alexandersen *et al.*, 2003b). This is in contrast to vaccination, with current FMDV vaccines prepared with inactivated virus and adjuvants, providing short term duration of SNTs and protection (Doel, 2005). Long-term maintenance of elevated, specific antibody titres in mice following acute VSV infection has been shown to be associated with the co-localisation of antigen with specific memory B cells within long-lived germinal centres (Bachmann *et al.*, 1996). VSV is a cytolytic

virus that does not persist in an infectious form in mice, thus highlighting the function of FDC trapping and retention serving as a long-term repository of immunogenic antigen for maintenance of SNTs. Hence, efficient retention within the germinal centres of intact viral capsids, as opposed to the constituent viral proteins, may be a requirement for sustaining antibody responses relevant for providing protection against challenge. Indeed, in a recent review of the functional significance of antigen retained on FDCs, Kosco-Vilbois suggests the observation that B-cell responses are independent of FDC-associated antigen is only valid in mice that are immunised with forms of antigen that leave persistent depots (Kosco-Vilbois, 2003). Therefore, we believe that long-term antibody responses detectable after FMDV infection are maintained in part by antigen persisting on FDCs. Based on the evidence presented here we suggest the persistence of FMDV after acute infection is both a consequence of the host immune response and a requirement for the long-term maintenance of protective virus-specific antibody responses.

3. FMDV can induce a specific and rapid CD4⁺ T-cell-independent neutralising isotype class switched antibody response in naïve cattle

3.1. Introduction

Experimental FMDV infection is characterised by a short incubation period of 1 to 3 days followed by pyrexia, formation of vesicles and a short viraemic phase with clinical resolution and virus clearance coinciding closely with the emergence of serum neutralising antibodies (Alexandersen *et al.*, 2003b). There is a close correlation between protection from disease after recovery from infection or after immunisation and the titre of circulating antibodies (Alexandersen *et al.*, 2003b). However, ruminants exposed to virus, whether vaccinated or not can carry FMDV in the oropharynx for years, following resolution of the acute infection (Alexandersen *et al.*, 2002). Because of their importance, a number of studies have examined the classes and subclasses of circulating neutralising antibody. Specific IgM has been detected in the serum from 3 to 7 days post-infection and specific IgG1 and IgG2 have been detected from 4 days post-infection (Doel, 2005, Salt *et al.*, 1996a) with neutralising titres of circulating antibody persisting up to 4.5 years post-infection (Cunliffe, 1964).

In contrast to the well defined role of humoral immune responses, the contribution of T-cell-mediated responses to immunity and their role in the induction of protective B-cell responses to FMDV in the natural host species are poorly understood. Observations in murine infection models indicate that acute cytopathic viral infections frequently induce T-I antibody responses. It has been proposed that such rapid antibody responses are required to facilitate control of virus spread through the

circulation and to ensure host survival, in contrast to non-cytopathic viruses like LCMV in mice where initial control is largely dependent on cytotoxic T lymphocyte responses, as opposed to neutralising antibody (Bachmann and Zinkernagel, 1997, Fehr *et al.*, 1996, Lee *et al.*, 2005). The kinetics of the early antibody response to FMDV is consistent with the responses seen for other rapidly replicating cytolytic viruses. The example of VSV in mice demonstrates an early T-I B-cell response where circulating, neutralising IgM can be detected as early as 48 hours post-infection followed by a rapid and efficient switch to a long-lived and protective IgG response (Bachmann and Zinkernagel, 1997, Hangartner *et al.*, 2006). Borca *et al.* reported that the protective immune response against FMDV in a murine experimental model was T-I (Borca *et al.*, 1986). However, a role for T cells in the induction of antibody responses in ruminants has been suggested, based on the demonstration of FMDV-specific CD4⁺ T-cell-proliferative responses following infection or vaccination with virus or peptide (Blanco *et al.*, 2001, Collen and Doel, 1990, Gerner *et al.*, 2007). Until recently, CD8⁺ T-cell responses to FMDV in livestock had only been demonstrated in infected animals, but the T-cell proliferation assays employed were unable to demonstrate whether or not the detected responses were class I MHC-restricted (Childerstone *et al.*, 1999). Recently, Guzman *et al.* used IFN- γ production to demonstrate virus-specific MHC class I-restricted CD8⁺ T-cell responses in cattle infected or vaccinated with FMDV, but the role of these CD8⁺ T cells in immunity to FMDV infection is still not known (Guzman *et al.*, 2008). There is an abundant $\gamma\delta$ T cell population in ruminants, $\gamma\delta$ T cells make up between 10 to 15% of PBMC in adult cattle, with even greater numbers (up to 50%) reported in juvenile animals (Clevers *et al.*, 1990, Pollock and Welsh, 2002). However, there is

no clear consensus on the role of these cells in immunity to infections in ruminants. Most of the $\gamma\delta$ T cells in the blood of young ruminants express WC1, a molecule shown to modulate $\gamma\delta$ T cell activation (Hanby-Flarida *et al.*, 1996, Pillai *et al.*, 2007, Takamatsu *et al.*, 1997), whereas many of the $\gamma\delta$ T cells in lymphoid tissues are WC1⁻ (MacHugh *et al.*, 1997). FMDV vaccine antigen has been shown to induce proliferation and cytokine production in naïve pig $\gamma\delta$ T cells, suggesting that these cells could contribute to the early immune response to FMD vaccination (Takamatsu *et al.*, 2006).

The three major subpopulations of bovine T lymphocytes identified in the circulation and secondary lymphoid organs of cattle can be effectively depleted *in vivo* by administering the appropriate mouse MAbs (Howard *et al.*, 1989, Naessens *et al.*, 1998). Administering relatively low doses (0.1 to 0.3 mg/kg) of MAbs to calves has been shown to effectively deplete peripheral blood and spleen T-lymphocyte populations but sparse numbers of target cells have been shown to persist in the lymph nodes at these doses (Naessens *et al.*, 1998). Administering anti-CD4 MAbs at this low dose range to cattle has been shown to significantly alter the host response to pathogens, for example, CD4 depletion during bovine virus diarrhoea virus infection resulted in extension of the duration of viraemia and an increase in titre of the virus in blood (Howard *et al.*, 1992). Similar doses administered during respiratory syncytial virus infection in calves increased the extent of pulmonary lesions and suppressed the antibody response (Naessens *et al.*, 1998, Taylor *et al.*, 1995, Thomas *et al.*, 1996). In addition, a possible role of $\gamma\delta$ T cells in the immune response to the intracellular pathogen *Mycobacterium bovis* has been demonstrated in calves

depleted of WC1⁺ cells, using a WC1-specific mouse MAb at this low dose range (Kennedy *et al.*, 2002). Depletion of peripheral lymph node T lymphocytes is difficult to achieve, doses of 2mg/kg are required to deplete CD4⁺ T cells from these tissues (Naessens *et al.*, 1998). Depletion of circulating CD8⁺ T cells is also difficult to achieve (Howard *et al.*, 1989, Howard *et al.*, 1992). Partial depletion, at relatively low doses has been shown to significantly influence the host immune response to pathogens, for example, administering 20mg of anti-CD8 MAb in total to 6 day old calves over a 5 day period has been shown to induce partial depletion of circulating CD8⁺ cells. The partially depleted calves excreted significantly more rotavirus than the control calves, implying a role for CD8⁺ cells in limiting primary rotavirus infection (Oldham *et al.*, 1993). In addition, incomplete CD8⁺ cell depletion with higher doses of anti-CD8 MAbs administered to approximately 9 day old calves (40 mg MAb in total administered over 10 days) demonstrated that CD8⁺ T cells play a dominant role in recovery from respiratory syncytial virus infection (Taylor *et al.*, 1995). Nasopharyngeal excretion of respiratory syncytial virus was prolonged in calves depleted of CD8⁺ cells, the depleted calves also presented more severe pulmonary lesions and virus could be isolated from lung washes for a longer period compared to the controls.

3.2. Aims of the chapter

To determine if CD4⁺, CD8⁺ or WC1⁺ T lymphocytes play a dominant role in the resolution of FMDV infection in naïve calves.

This was investigated by:

- ◇ the application of T lymphocyte depletion protocols in calves using subset specific mouse MAbs to deplete either CD4⁺, CD8⁺ or WC1⁺ T cells during the early stages of infection with FMDV
- ◇ monitoring the extent of T-cell depletion from the circulation and from peripheral lymph nodes
- ◇ comparing clinical FMD progression compared to control, non-depleted animals
- ◇ monitoring virus clearance by quantitative rRT-PCR and by virus isolation
- ◇ monitoring the virus neutralising antibody response
- ◇ analysing the profile of the FMDV-specific antibody isotype response
- ◇ monitoring the antibody response to viral non-structural proteins and G-H loop peptides

3.3. Materials and methods

3.3.1. Experimental procedures

Animal experiments were carried out at the Institute for Animal Health under project licence number PPL70/6212 as described under section 2.3.1. A total of 12 cattle, 2 to 4 months of age, were used in the studies. In an initial experiment, eight cattle were allocated into 4 pairs, each of which received anti-CD4, anti-CD8, anti-WC1 or

an isotype-matched control MAb over a period of 3 days, starting the day before virus challenge. Doses of 3mg, 21.5mg and 21mg diluted in PBS (CSU, IAH) were administered intravenously to each calf on days -1, 0 (challenge day) and 1 respectively, giving a total dose of approximately 0.76mg of antibody per kg body weight. In a second experiment, 4 cattle were divided into pairs that received either anti-CD4 or a control MAb over a 4 day period starting 2 days before challenge. The animals were given 20mg of MAb on day -2 and 45mg on each of the following 3 days, giving a total dose of approximately 2.58mg of antibody per kg body weight. Cattle were challenged with FMDV by subepidermo-lingual injection of 0.2ml of 10^5 TCID₅₀ into each of two sites with the cattle-adapted type O UKG 34/2001 strain of virus (section 2.3.1.1). Clinical observations were conducted daily and scored until resolution of disease. The right prescapular lymph node was removed from animals in the second experiment five days post-challenge, under sedation and local anaesthetic. Clotted blood and heparinised blood were collected at intervals throughout the study and at post-mortem on day 30 for animals in experiment 1 and on day 29 for animals in experiment 2. Mandibular lymph node and probang samples were collected at post-mortem.

3.3.2. Clinical scoring system

Clinical signs of FMD and rectal temperatures were scored as described in Table 6, using a modified subjective scoring system based on a method described previously (Quan *et al.*, 2004). Cattle could score a maximum of 22 points, with the sum of the coronary band lesions divided by 2 to prevent the clinical score being dominated by foot lesions.

Table 6. Clinical scoring system.

Clinical signs	Clinical score *
Coronary band lesions **	0 = none 1 = elevated temperature/congestion or healing vesicle 2 = vesicle 3 = severe lesion (up to detachment of heal or equivalent)
Tongue lesions	0 = none 1 = elevated temperature/congestion or healing vesicle 2 = vesicle 3 = severe lesion
Dental pad, oral cavity or muzzle (nose and mouth) lesions	0 = none 1 = elevated temperature/congestion or healing vesicle 2 = vesicle 3 = severe lesion
Teat or udder lesions ***	0 = none 1 = elevated temperature/congestion or healing vesicle 2 = vesicle 3 = severe lesion
Lameness	0 = none 1 = lame 2 = recumbent
Nasal discharge	0 = none 1 = serous 2 = sero-necrotic 3 = necrotic
Rectal temperature	0 = temperature < 39.5°C 1 = temperature ≥ 39.5°C to < 40°C 2 = temperature ≥ 40°C

* Cattle could score a maximum of 22 points.

** Coronary band lesions were scored for each foot. The sum of the coronary band lesion scores were divided by 2 to prevent the clinical scores being dominated by foot lesions.

*** All experimental animals were male.

3.3.3. Mouse monoclonal antibodies used for depletion

The MAbs used for depletion, which are described in the proceedings of the First International Workshop on Bovine, Sheep, and Goat leukocyte Differentiation Antigens (Howard and Morrison, 1991), were CC8 (anti-CD4), IL-A11 (anti-CD4), CC63 (anti-CD8) and CC15 (anti-WC1). MAb TRT3 raised against turkey rhinotracheitis virus was administered to control animals (Cook *et al.*, 1993). During

experiment 1, anti-CD4 treated animals received MAb CC8 only, whereas during experiment 2, anti-CD4 treated animals received a combination of CC8 and IL-A11. All MAbs were murine IgG2a, and all of the hybridomas were produced at the IAH, except IL-A11 which was provided by the International Livestock Research Institute, Nairobi. MiniPERM (Sigma-Aldrich, UK) hybridoma tissue culture supernatants, prepared with pre-absorbed serum, were kindly provided by B Jones, IAH. HiTrap Protein G HP columns (Amersham Biosciences, UK) were used for purification and dialysis membrane bags (Medical International, UK) were used for dialysis in Ca/Mg free PBS (CSU, IAH) to desalt the eluate. An Ultraspec 2001 Pro spectrophotometer (Biochrom, UK) was used for protein quantification and Vivaspin 15R columns (Sartorius, UK) were used to concentrate the sample if required.

3.3.4. Preparation of mononuclear cells from tissue and blood

Mononuclear cells were prepared from samples of prescapular lymph nodes by slicing the tissue into small fragments which were gently teased apart using forceps and a needle in PBS (CSU, IAH) containing 5% (v/v) fetal calf serum (Autogen Bioclear, UK). The tissue fragments were then disrupted through sterile gauze with a syringe. Viable mononuclear cells were isolated from these lymph node suspensions and from heparinised peripheral blood by diluting them with an equal volume of PBS and underlaying them with 13ml Histopaque 1077 (Sigma-Aldrich, UK) before centrifugation at 1000×g for 30 minutes at 18°C with the centrifuge brake off. Cells at the interface were collected, washed three times by dilution in PBS and centrifugation at 250×g for 8 minutes at 8°C. Cells were counted on a haemocytometer (Assistant, Germany) and their viability assessed by trypan blue

staining (Sigma-Aldrich, UK). Cells were subsequently analysed by flow cytometry and additional aliquots were stored at -80°C in 10% (v/v) dimethylsulphoxide (Sigma-Aldrich, UK) in fetal calf serum.

3.3.5. Flow cytometry

Blood mononuclear cells (M Windsor and L Reid, IAH, kindly assisted with the analysis) were analysed by flow cytometry to evaluate the degree of lymphocyte depletion, using the following MAbs: CC30 (anti-CD4), CC58 (anti-CD8) and CC39 (anti-WC1) (Howard and Morrison, 1991). MAb CC37 (anti-CD21) was used as a positive control and MAb TRT1, raised against turkey rhinotracheitis virus, as an isotype-matched negative control (Cook *et al.*, 1993, Howard and Morrison, 1991). Lymph node mononuclear cells were analysed by L Reid (IAH) by flow cytometry using MAbs CC30 to evaluate the degree of CD4 depletion in combination with positive control MAb CC37 and negative control MAb TRT1. All MAbs were murine IgG1 produced at the IAH.

Cell suspensions were stained with MAbs to detect surface proteins by flow cytometry as described under section 2.3.20.1. A minimum of 10000 viable cells were analysed in each sample, in addition, 100000 viable PBMC were analysed on day 1 in duplicate in experiment 1 and on days 0 and 4 in triplicate in experiment 2 to assess CD4^{+} T-cell depletion.

Preliminary studies, using blood and lymph node mononuclear cells from non-infected animals, were undertaken to determine if the MAbs used for depletion

blocked the staining of MAbs of the respective specificities used for evaluating the degree of lymphocyte depletion. Mononuclear cells were prepared from samples of prescapular lymph nodes and heparinised blood as described under section 3.3.4. Approximately 3×10^5 cells per well were placed into U bottom 96 microwell plates (Sigma-Aldrich, UK). The cells were pelleted by centrifugation at $250 \times g$ for 4 min at 8°C and resuspended in complete RPMI media (CSU, IAH) containing 10% (v/v) fetal calf serum (Autogen Bioclear, UK). The cells were incubated with the MAbs used for depletion (section 3.3.3) for 1 hour or for 20 hours at 37°C . After the incubation period, cells were washed with FACS wash buffer (Appendix 1) and stained with the IgG1 MAbs, used for evaluating the degree of lymphocyte depletion, diluted in FACS wash buffer (section 2.3.20.1). Cells were subsequently washed twice before incubation with goat anti-mouse IgG2a and IgG1 specific secondary MAb (Alexa fluor, Molecular Probes, UK) for 15 minutes at room temperature in the dark for flow cytometry analysis (section 2.3.20.1).

3.3.6. Immunofluorescence confocal microscopy

Prescapular lymph node samples were snap frozen in cryomatrix (Sakura Finetek, NL) and stored at -80°C until processed. Ten approximately $7\mu\text{m}$ thick acetone fixed cryosections from different regions of the prescapular lymph nodes of each animal were labelled (section 2.3.17.1) with the following murine MAbs: CC30 (anti-CD4), MM1A (anti-CD3, IgG1), CC51 (anti-CD21, IgG2b) (Howard and Morrison, 1991) and isotype-matched control MAbs TRT1 and AV29 (a MAb directed against chicken CD4 antigen, IgG2b) (Kwong *et al.*, 2002). Acetone fixed cryosections of mandibular lymph nodes were labelled with IB11, a murine MAb shown to be

specific for conformational, non-neutralising epitopes of the FMDV capsid (Juleff *et al.*, 2008) in combination with CC51, a dark zone follicular dendritic cell marker D46 (anti-ovine fibrinogen, IgG2a) (Lefevre *et al.*, 2007) and isotype-matched control MAbs TRT1, TRT3 (IgG2a) (Cook *et al.*, 1993) and AV29 (Table 1). All MAbs used for confocal microscopy were produced at the IAH. Goat anti-mouse Molecular Probes Alexa-Fluor-conjugated secondary antibodies (Invitrogen, UK) were used for detection and as a control in the absence of primary antibody. Stack images were analysed to detect CD4⁺ T-cell depletion. All data were collected sequentially using a Leica SP2 scanning laser confocal microscope.

Preliminary studies, on 7µm thick cryosections of prescapular lymph node harvested from non-infected animals, were undertaken to determine if the MAbs used for depletion blocked the staining of MAbs used for analysis. Specifically to determine if the reactivity of MAb CC30 used to evaluate the degree of CD4⁺ depletion in experiment 2, was blocked by the MAbs CC8 and IL-A11 used for depletion. Immunofluorescence labelling was performed as described under section 2.3.17.1. Sections were incubated with the MAbs used for depletion for 30 minutes at room temperature. Slides were washed 5 times with Ca/Mg free PBS (CSU, IAH) and incubated with the MAbs used for detection for 30 minutes at room temperature. Slides were washed 5 times in Ca/Mg free PBS and incubated with the secondary goat anti-mouse IgG2a and IgG1 secondary antibodies (Alexa fluor, Molecular Probes, UK) at a working dilution of 1:500 for 20 minutes in the dark, washed and mounted as described under section 2.3.17.1.

3.3.7. Quantitative real-time reverse transcription-polymerase chain reaction

Total nucleic acid was extracted from serum and probang samples using a MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, UK) and MagNA Pure LC robot (Roche, UK) (Shaw *et al.*, 2007, Shaw *et al.*, 2004). Two hundred µL of sample was added to 300µL of Lysis/Binding Buffer (Roche, UK). The lysate was mixed by pipetting and transferred to a sample cartridge in the MagNA Pure LC robot.

Genomic DNA was removed by DNase 1 treatment (Roche, UK) and purified RNA eluted with 50µL Roche Elution Buffer. A quantitative rRT-PCR method specific for FMDV O UKG 34/2001 was used to quantify the FMDV genome copies in serum and in probang samples as described under section 2.3.10. Fifty PCR cycles were carried out and samples that did not have a detectable signal above threshold after 50 cycles were taken to be negative (Quan *et al.*, 2004). Samples with threshold cycle values greater or equal to 39 were designated 'borderline' and were subsequently retested to confirm their positive/negative status (Reid *et al.*, 2003).

3.3.8. Virus isolation and antigen detection ELISA

The presence of virus in serum and in probang samples was determined by inoculation of monolayers of primary BTY cells (Snowdon, 1966) with 200µL of sample and examination for cytopathic effect 24, 48 and 72 hours post-inoculation as described under section 2.3.21.4. An ELISA, kindly performed by G Hutchings (IAH) was used to confirm the presence of FMDV (Ferris and Dawson, 1988).

3.3.9. Virus neutralising antibody test

Serum samples were examined for anti-FMDV neutralising antibodies as described in the Office International des Epizooties (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 5th edition, 2004 (Golding *et al.*, 1976). The tests were performed under the guidance of P Hamblin, IAH. Serum was inactivated at 56°C for 1 hour before testing. Starting from a ¼ dilution, sera were diluted in serum free medium in a two-fold, dilution series across flat-bottomed Nunc TC microwell 96 FSI plates (Fisher Scientific, UK) in duplicate wells at a volume of 50µL. Fifty µL of titrated O UKG virus stock provided by P Hamblin, IAH (containing approximately 1×10^2 TCID₅₀ as titrated on a virus control plate) was added to each well and plates were incubated at 37°C for 1 hour. A cell suspension at 1×10^6 IB-RS-2 cells per mL was made up in medium containing 10% (v/v) fetal calf serum (Autogen Bioclear, UK). Fifty µL of the cell suspension (0.5×10^5 cells) was added to each well. Duplicate wells containing cells with negative serum (kindly provided by P Hamblin, IAH), serum free medium (also used for diluting the virus stock) and medium were included on the plates as cell controls. Reference serum control plates containing standard 21-day convalescent serum (kindly provided by P Hamblin, IAH) were run in parallel with test plates. The plates were incubated at 37°C with readings taken at 24, 48 and 72 hours for cytopathic effect. After 72 hours the plates were stained with 0.4% (w/v) naphthalene black (Searle Diagnostics, UK) in PBS (CSU, IAH) containing 8% (w/v) citric acid crystals (Sigma-Aldrich, UK). Positive wells (where the virus has been neutralised and the cells remain intact) were seen to contain blue-stained cell sheets, negative wells were empty. Titres were expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells

were protected (Karber, 1931). The tests were considered valid when the cell sheets in the cells controls were intact and the reference serum was within twofold of its expected titre. Sera with titres greater than or equal to 1/45 were considered positive (Golding *et al.*, 1976).

3.3.10. 3ABC non-structural protein ELISA

Serum samples were examined for the presence of antibodies directed against the non-structural 3ABC protein of FMDV, using the commercially available Ceditest FMDV-NS blocking ELISA (Cedi-Diagnostic, NL). The test was performed with negative, weak positive and positive controls supplied with the kit in duplicate and the test serum samples were analysed in triplicate. The OD was read at 450nm on a MRX Dynex Technologies reader (Dynex, UK). Samples were considered positive if the percentage inhibition was ≥ 50 (Sorensen *et al.*, 1998).

3.3.11. Isotype-specific ELISA for the detection of anti-FMDV antibodies

An anti-FMDV sandwich ELISA was used to measure specific IgG1, IgG2 and IgM in serum samples (Mulcahy *et al.*, 1990). The test samples were analysed with M Windsor, IAH. Ninety-six-well Maxisorb Nunc Immunoplates (Sigma-Aldrich, UK) were coated overnight at 2 to 8°C with a 50µL solution of rabbit anti-FMDV serotype-specific hyperimmune antiserum (kindly provided by N Ferris, IAH) diluted 1:5000 in 0.1M carbonate/bicarbonate buffer (CSU, IAH). Coated plates were washed 4 times in 0.05% (v/v) Tween-20 (Sigma-Aldrich, UK) in PBS (CSU, IAH) then incubated with 50µL of pre-titrated inactivated O1 Manisa FMDV whole viral antigen in excess (kindly provided by N Ferris, IAH). This step and all subsequent

incubation steps were carried out at 37°C for 1 hour. Plates were washed and blocked with a 1mg/mL solution of sodium casein (Sigma-Aldrich, UK) in PBS. Test serum and antibodies were diluted in the sodium casein solution. Plates were washed and 50µL of duplicate threefold dilution series of each serum sample were added at a starting dilution of 1/50. Antibody isotypes were detected with 50µL of a 1/500 dilution of MAbs to bovine IgG1 (B37), IgG2 (B192) and IgM (B67) obtained from the Department of Veterinary Medicine, Bristol University. This was followed by incubation with 50µL of a 1/1000 dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG (DakoCytomation, UK). After a final wash, plates were incubated at room temperature with 50µL of OPD substrate (Sigma, UK) diluted in H₂O (CSU, IAH). The reaction was stopped with 50µL of a 1.84M solution of sulphuric acid (Sigma-Aldrich, UK). To avoid competition between IgM and IgG, all samples destined for anti-IgM analysis were first absorbed on plates coated with goat anti-bovine IgG (1mg/ml, Southern Biotech, UK) then transferred to the viral antigen coated plates. The OD was read at 492nm on a MRX Dynex Technologies reader (Dynex, UK). Wells were only considered positive if they were greater than 1.5 times the mean background OD for that dilution. Antibody titres were expressed as the reciprocal of the last positive dilution.

3.3.12. Indirect peptide ELISA

Serum samples from animals in both experiments receiving anti-CD4 or TRT3 MAbs were examined for the presence of antibodies directed against the VP1₁₃₅₋₁₅₆ G-H loop on the surface of FMDV capsids (M Windsor, IAH, kindly assisted with the analysis). A peptide encompassing amino acid residues 135 to 156 of FMDV O UKG

34/2001 (KYGESPVNTNVRGDLQVLAQKAA) was kindly produced by L Hunt, IAH. A second peptide, kindly provided by V Fowler, IAH, encompassing the same residues of FMDV O1BFS (RYSRNAVPNLRGDLQVLAQKVA) was used for analysis to confirm that our results were consistent with previously published data (Fowler *et al.*, 2008). The indirect peptide ELISA was performed as previously described (Fowler *et al.*, 2008) with modifications. Ninety-six-well Maxisorb Nunc Immunoplates (Sigma-Aldrich, UK) were coated overnight at 4°C with 100µL/well peptide (at a concentration of 4µg/mL for O UKG peptide and 2µg/mL for O1BFS peptide) (Fowler *et al.*, 2008) in PBS (CSU, IAH), washed 4 times in 0.05% (v/v) Tween-20 (Sigma-Aldrich, UK) in PBS and blocked with PBS containing sodium casein (Sigma-Aldrich, UK) at 1mg/mL. This step and all subsequent incubation steps were carried out at 37°C for 1 hour. Sera were added in duplicate at 50µL per well starting at 1/50 with tripling dilutions in PBS sodium casein, incubated, washed and detected with horseradish peroxidase-conjugated goat anti-bovine IgG (Southern Biotech, UK). Plates were washed and visualised with OPD substrate (Sigma-Aldrich, UK) diluted in H₂O (CSU, IAH). Reactions were stopped with 1.84M sulphuric acid (Sigma-Aldrich, UK) and absorbance read at 490nm on a MRX Dynex Technologies reader (Dynex, UK). Wells were only considered positive if they were greater than 1.5 times the mean background OD for that dilution. Antibody titres were expressed as the reciprocal of the last positive dilution.

3.3.13. Statistical analysis

Statistical analysis was performed under the guidance of S Gubbins, IAH. To investigate the effect of immune cell depletion on the titres of FMDV-specific

antibody measured by the Ig isotype-specific ELISA, a Gompertz function was used to describe the antibody titre, Y , as a function of time,

$$\log_{10}(Y) = \kappa \exp(-\exp(-\beta(t - \delta))),$$

Where κ is the upper asymptote (i.e. maximum titre), β is the rate of increase in titre and δ is the delay parameter. The parameters (κ , β and δ) were estimated using the least-squares regression. Parallel curve analysis (Ross, 1990) of the data from individual animals was used to identify significant ($p < 0.05$) differences in the parameters amongst treatment groups (i.e. TRT3, anti-WC1, anti-CD4 and anti-CD8 groups), starting from a model in which all parameters differed amongst animals.

The analysis was performed using MATLAB (MathWorks, USA). The non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) was used to test the hypothesis that the different treatment groups had the same distribution of onset of virus neutralising antibody titres post-infection. The analysis was performed using the R Project for Statistical Computing. The ANOVA general linear model (Lindman, 1974) was used to determine if there was a statistically significant association between the peak level of viraemia measured by quantitative rRT-PCR, expressed as genome copies per mL serum, and the treatment group (i.e. TRT3, anti-WC1, anti-CD4 and anti-CD8 groups). Minitab software (Minitab Limited, UK) was used to perform the analysis.

3.4. Results

3.4.1. Efficiency of T cell subset depletion

In preparation for the *in vivo* T-cell depletion studies, the potential cross reactivity between the MAbs used for depletion and those used for detection was investigated. Flow cytometry (Figure 46) and immunofluorescence confocal microscopy (Figure 47 and Figure 48) studies, using blood and lymph node mononuclear cells and prescapular lymph node cryosections from non-infected animals confirmed that the MAbs used for depletion did not block the staining of MAbs of the respective specificities used for evaluating the degree of lymphocyte depletion.

Administration of anti-CD4 MAbs resulted in a rapid reduction in the percentage of circulating CD4⁺ cells within 24 hours, from 11% and 10.8% to 0.15% and 0.17% respectively for the 2 animals (RZ53 and RZ54) in experiment 1, and from 18.4% and 26% to 0.02% and 0.17% for the 2 animals (VT74 and VT75) in experiment 2. This depletion was confirmed by analysis of 100000 viable cells in duplicate, collected from experiment 1 animals on day 1 post-infection (RZ53 = 0.04 and 0.05% CD4⁺ cells and RZ54 = 0.04 and 0.04% CD4⁺ cells) and in triplicate for experiment 2 animals on days 0 and 4 post-infection (Day 0: VT74 = 0.05% [± 0.02] and VT75 = 0.04% [± 0.01] CD4⁺ cells. Day 4: VT74 = 0.06% [± 0.01] and VT75 = 0.03% [± 0.01] CD4⁺ cells. Values expressed as mean [\pm standard deviation]). Depletion was maintained for 7 days post-infection with percentages of CD4⁺ cells consistently below or equal to background nonspecific binding detected with the isotype control MAbs, after which the numbers of CD4⁺ cells gradually increased (Figure 49, Table 1). A similar level and duration of depletion was observed

following treatment with anti-WC1 MAb, the numbers of circulating WC1⁺ cells in the two animals (RZ51 and RZ52) in experiment 1 decreased from 11.7% and 22.3% on day -1 to 0.06% and 0.06% on day 0 respectively and maintained at these low levels until day 7 (Figure 49, Table 7). In contrast, treatment with anti-CD8⁺ MAb resulted in more gradual and only partial depletion; the numbers of circulating CD8⁺ cells in the two treated animals (RZ55 and RZ56) decreased from 4.0% and 9.4% on day -1, to 3.1% and 5.7% on day 0, and to 1.3% and 4% on day 7 respectively (Figure 49, Table 1). During acute infection in the anti-CD4, anti-WC1 and anti-CD8 MAb treated animals, there were no major changes in the proportion of the T-cell subsets not targeted for depletion or CD21⁺ B cells, consistent with the specificity of these MAbs (Figure 50 and Figure 51) (Howard and Morrison, 1991). In addition, there were no major changes in the proportions of the T-cell subsets (or CD21⁺ B cells) in animals receiving the control antibody during acute infection, although some fluctuation in the percentage representation of each subset was observed during the course of the studies (Table 1, Figure 49 and Figure 52).

Immunohistological examination of prescapular lymph nodes surgically removed from experiment 2 animals on day 5 post-infection, demonstrated the absence of CD4⁺ cells throughout the node (although CD3⁺ cells were still readily detectable), including the cortex and follicles, paracortical area, and the medullary cords and sinuses of both anti-CD4 MAb treated animals (Figure 53 to Figure 55). These analyses used both 10 separate sections and stacking of images from the confocal microscopy examinations, to confirm that the CD4⁺ cell depletion was indeed throughout the node. These findings were supported by flow cytometry analysis of

lymph node cell suspensions (kindly performed by L Reid, IAH), in which the percentages of CD4⁺ T cells were comparable to that detected with the isotype control MAbs.

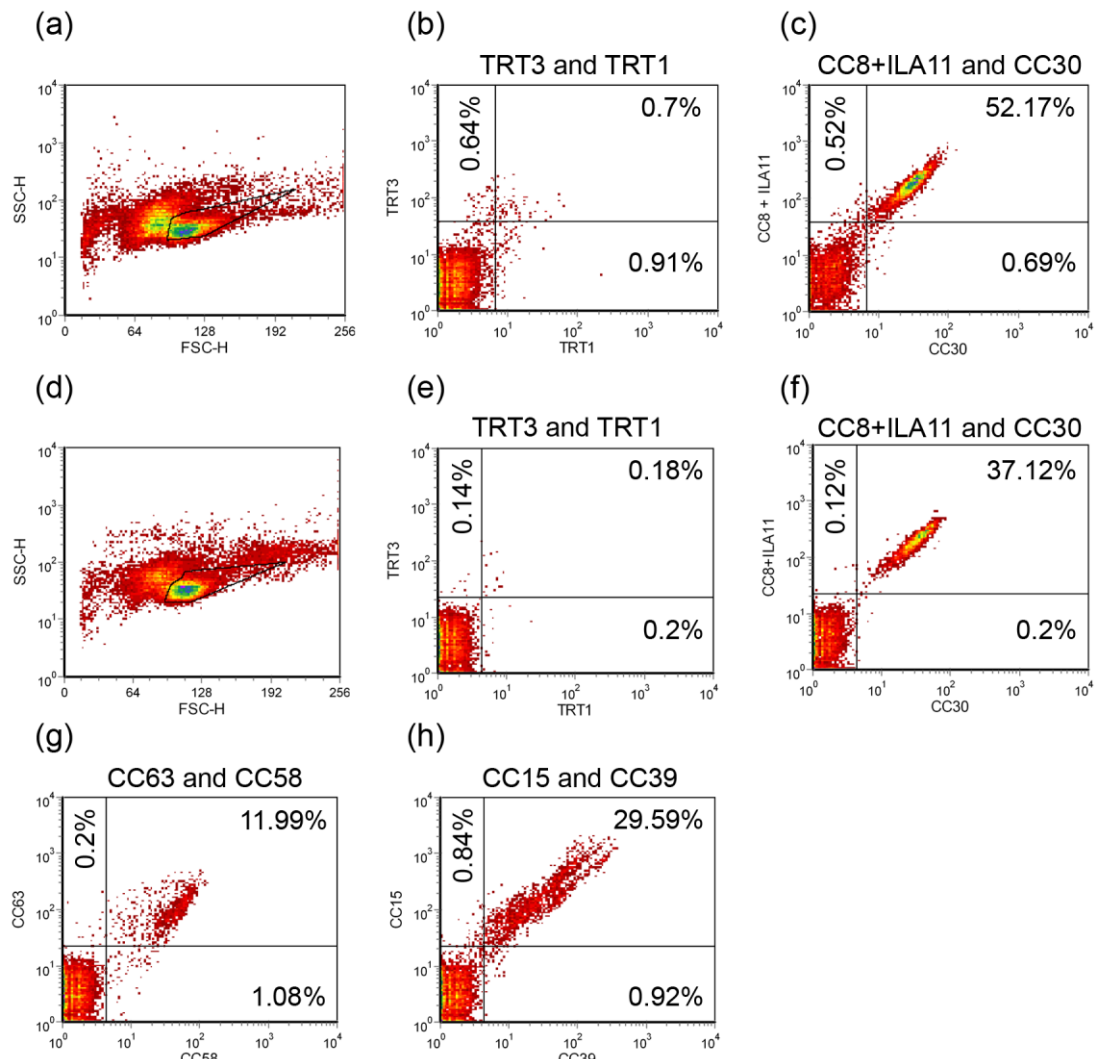


Figure 46. The MAbs used for depletion did not block the staining activity of MAbs of the respective specificities used for evaluating the degree of lymphocyte depletion by flow cytometry.

(a) to (c) Prescapular lymph node and (d) to (h), PBMC from a non-infected control animal evaluated by flow cytometry. The cells were incubated with the IgG2a MAbs used for depletion (section 3.3.3) for 20 hours at 37°C followed by staining with the IgG1 MAbs used to evaluate the degree of lymphocyte depletion (section 3.3.5). (a) and (d) Cells were gated on their forward scatter (FSC) and side scatter profiles (SSC), % represent the number of positive cells within the gate. (b) and (e) Background staining detected with isotype control MAbs TRT3 (IgG2a) and TRT1 (IgG1). (c) and (f) Anti-CD4 MAbs CC8 and IL-A11 (depletion MAbs) and CC30 (detection MAb). (g) Anti-CD8 MAbs CC63 (depletion MAb) and CC58 (detection MAb). (h) Anti-WC1 MAbs CC15 (depletion MAb) and CC39 (detection MAb). The MAbs used for depletion did not block the staining of MAbs of the respective specificities used for evaluating the degree of lymphocyte depletion after a 20 hour incubation period (these results were corroborated by flow cytometry analysis following an hour incubation period with the MAbs used for depletion, data not shown).

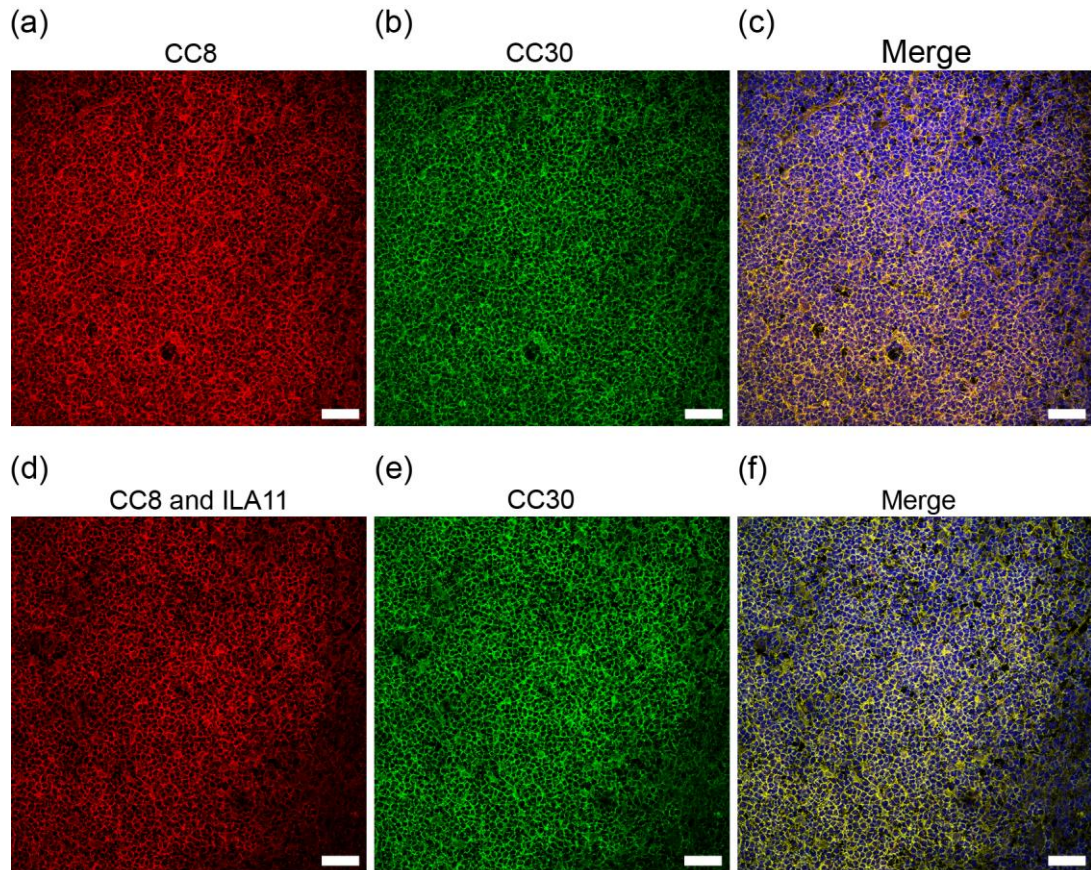


Figure 47. The anti-CD4 MAbs used for depletion did not block the staining activity of the anti-CD4 MAb used to evaluate the degree of lymphocyte depletion.

(a) to (f) Cryosections of the T cell zone of a prescapular lymph node harvested from a non-infected control animal. The cryosections were incubated with the anti-CD4 MAbs [(a) CC8 or (d) CC8 and IL-A11 (red)] used for depletion for 30 minutes followed by washing and incubation with the anti-CD4 MAb [(b) and (e) CC30 (green)] used for detection. (c) Merge image of (a) and (b). (f) Merge image of (d) and (e). Nuclei stained blue in merge images (DAPI), scale bars represent 40 μ m.

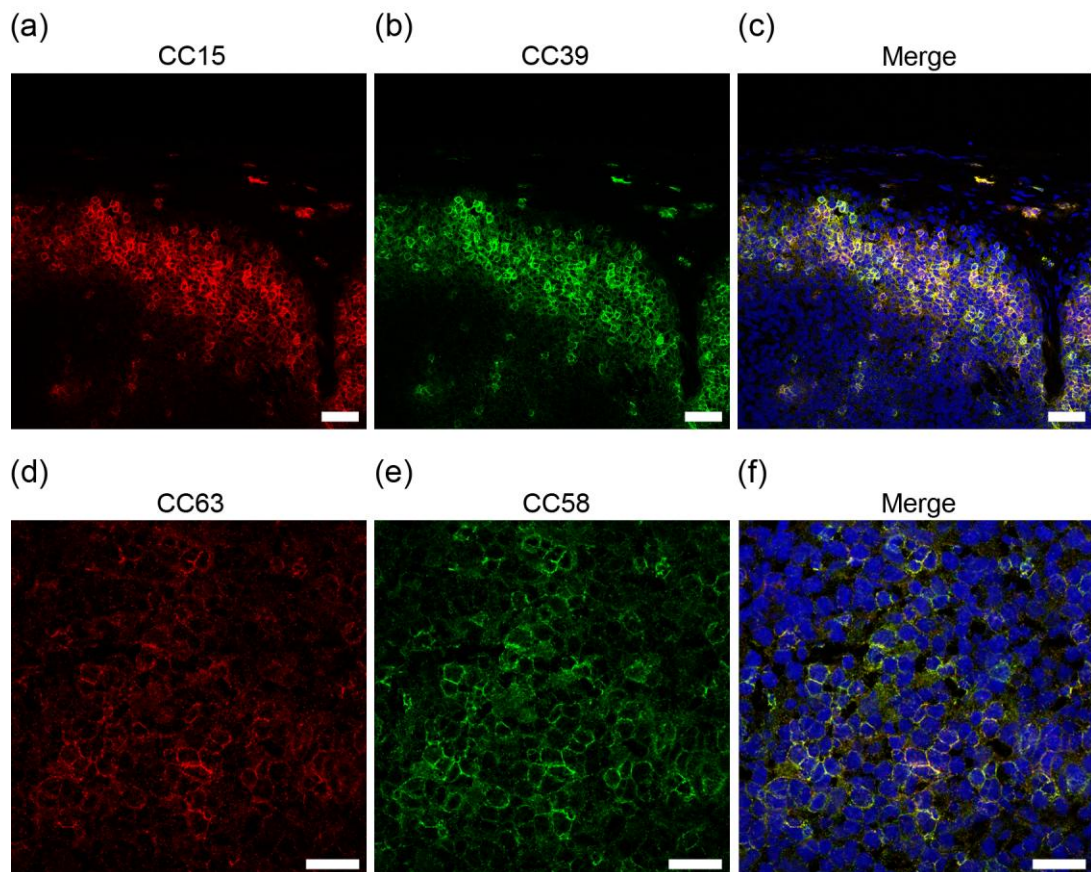


Figure 48. The anti-WC1 and anti-CD8 MAbs used for depletion did not block the staining activity of the MAbs of the respective specificities used for evaluating the degree of lymphocyte depletion.

(a) to (f) Cryosections of the cortex of a prescapular lymph node harvested from a non-infected control animal. The cryosections were incubated with the MAbs used for depletion [(a) anti-WC1 MAb CC15 or (d) anti-CD8 MAb CC63 (red)] for 30 minutes followed by washing and incubation with the MAbs used to evaluate depletion [(b) anti-WC1 MAb CC39 or (e) anti-CD8 MAb CC58 (green)]. (c) Merge image of (a) and (b). (f) Merge image of (d) and (e). Nuclei stained blue in merge images (DAPI), scale bars represent: (a) to (c), 40 μ m; (d) to (f), 20 μ m.

Table 7. Effect of MAb administration on the percentage of CD4⁺, WC1⁺ and CD8⁺ T-cell populations in peripheral blood measured by flow cytometry.

Animal	Cells targeted for depletion	Percentage CD4 ⁺ cells in peripheral blood*										
		Experiment 1. Days post-intradermolingual challenge.										
		-1	0	4	7	9	13					
RZ53	CD4	11.0	0.2	0.0	0.3	4.6	12.6					
RZ54	CD4	10.8	0.2	0.1	0.0	4.7	10.6					
RZ57	Control	15.7	16.2	9.0	13.1	14.9	20.3					
RZ58	Control	18.1	14.6	13.6	8.1	16.8	28.6					
Animal	Cells targeted for depletion	Percentage WC1 ⁺ cells in peripheral blood*										
		Experiment 1. Days post-intradermolingual challenge.										
		-1	0	4	7	9	13					
RZ51	WC1	11.7	0.1	0.1	0.2	0.9	1.2					
RZ52	WC1	22.3	0.1	0.0	0.4	1.7	2.3					
RZ57	Control	9.0	7.1	6.0	9.7	8.4	5.9					
RZ58	Control	19.9	13.8	8.9	12.3	14.6	10.4					
Animal	Cells targeted for depletion	Percentage CD8 ⁺ cells in peripheral blood*										
		Experiment 1. Days post-intradermolingual challenge.										
		-1	0	7	9	13						
RZ55	CD8	4.0	3.1	1.3	2.0	2.2						
RZ56	CD8	9.4	5.7	3.7	2.7	3.9						
RZ57	Control	7.4	6.4	8.1	6.4	6.9						
RZ58	Control	11.4	6.3	9.2	6.3	6.5						
Animal	Cells targeted for depletion	Percentage CD4 ⁺ cells in peripheral blood*										
		Experiment 2. Days post-intradermolingual challenge.										
		-2	-1	0	1	3	4	5	6	7	9	13
VT74	CD4	18.4	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.1	4.0
VT75	CD4	26.0	0.2	0.1	0.1	0.1	0.0	0.1	0.0	0.4	1.5	5.4
VT76	Control	20.0	17.7	19.1	10.7	17.3	15.0	19.8	20.2	18.8	21.8	16.6
VT77	Control	28.4	22.0	22.7	17.1	24.1	21.4	15.9	30.8	21.0	34.3	32.9

* A minimum of 10000 viable cells were analysed in each sample by flow cytometry, in addition, 100000 viable PBMC were analysed on day 1 in duplicate in experiment 1 and on days 0 and 4 in triplicate in experiment 2 to assess CD4⁺ T-cell depletion (see section 3.4.1). Percentages have been decreased to one decimal place. MAbs (anti-CD4 MAb CC8, anti-WC1 MAb CC15, anti-CD8 MAb CC63, control anti-turkey rhinotracheitis MAb TRT3) were administered to experiment 1 animals over three days starting the day before FMDV challenge. MAbs (anti-CD4 MAb CC8 and IL-A11, control anti-turkey rhinotracheitis MAb TRT3) were administered to experiment 2 animals over four days starting two days before FMDV challenge.

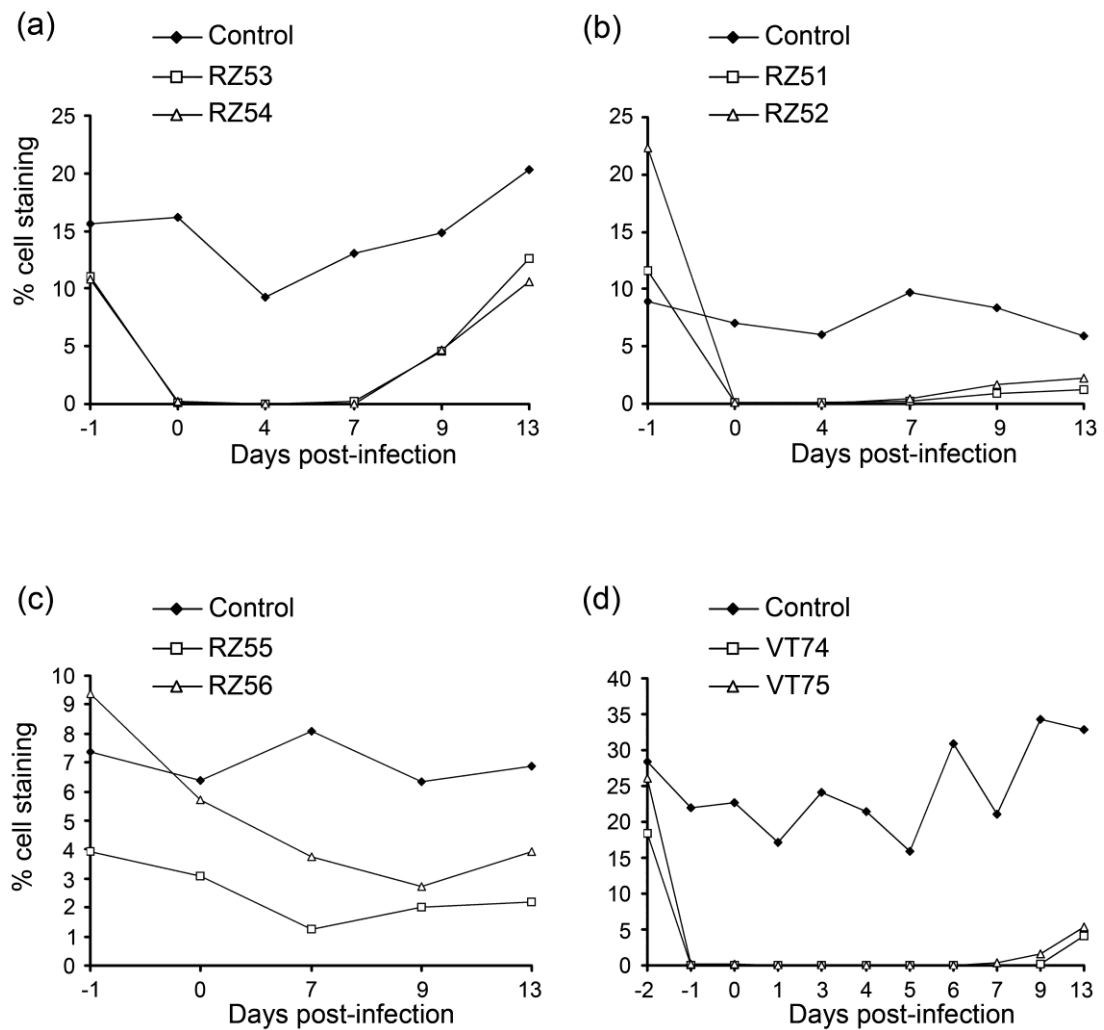


Figure 49. Effect of MAb administration on the percentage of T lymphocyte subpopulations in peripheral blood measured by flow cytometry.

(a) to (c) Experiment 1 animals, MAbs were administered over three days starting the day before FMDV challenge. (a) Percentage CD4⁺ cells in anti-CD4 MAb treated animals (RZ53 and RZ54) and a control animal (RZ57). (b) Percentage WC1⁺ cells in anti-WC1 MAb treated animals (RZ51, RZ52) and a control animal (RZ57). (c) Percentage CD8⁺ cells in anti-CD8 MAb treated animals (RZ55, RZ56) and a control animal (RZ57). (d) Experiment 2 animals. Percentage CD4⁺ cells in anti-CD4 MAb treated animals (VT74, VT75) and a control animal (VT77). MAbs were administered over four days starting two days before FMDV challenge.

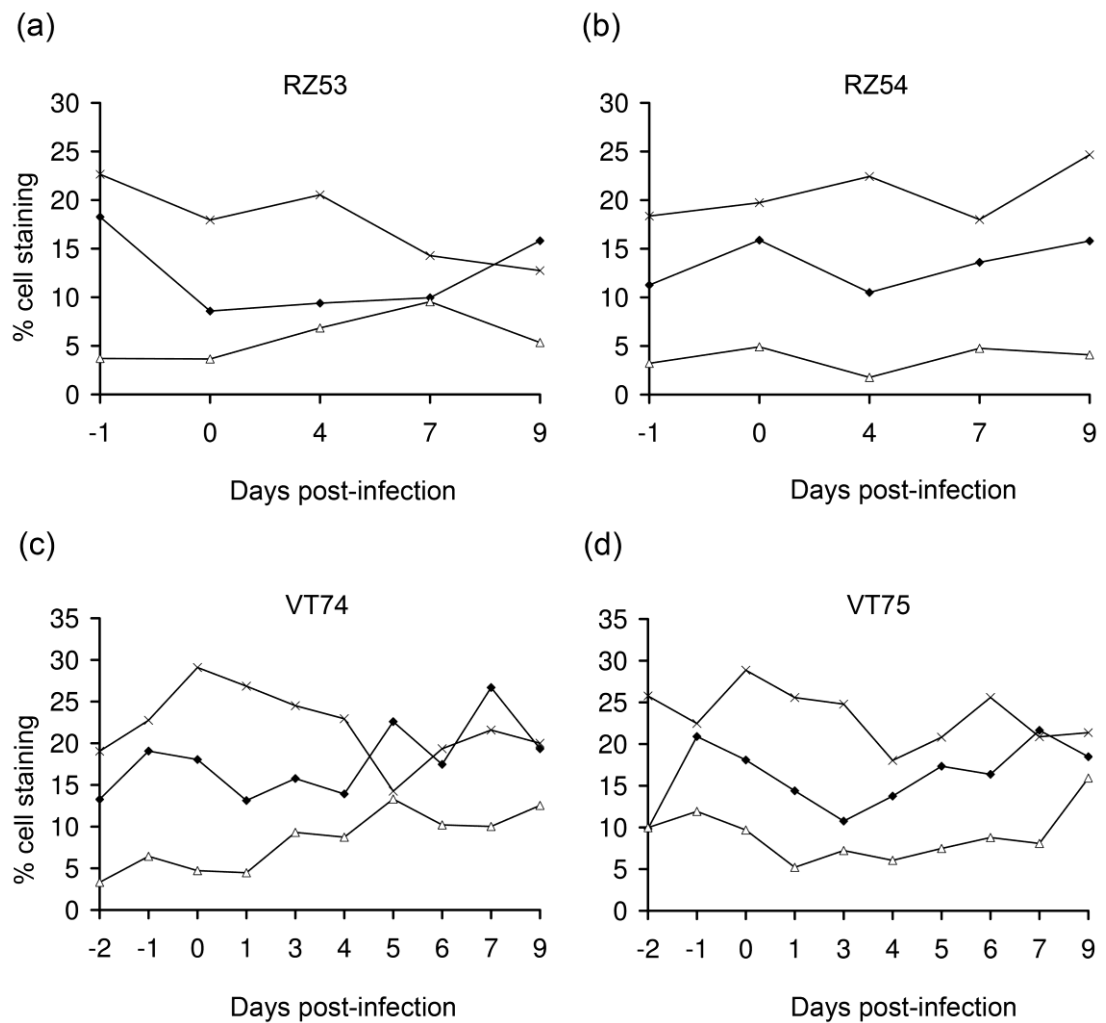


Figure 50. Effect of anti-CD4 MAb administration on the percentage of T lymphocyte subpopulation in the peripheral blood not targeted for depletion, measured by flow cytometry. (a) to (b) Experiment 1 animals, MAbs were administered over three days starting the day before FMDV challenge. (c) and (d) Experiment 2 animals, Mabs were administered over four days starting two days before FMDV challenge. ♦ = WC1⁺ cells, △ = CD8⁺ cells and × = CD21⁺ cells. Administering anti-CD4 MAbs did not result in non-specific depletion of other cell types.

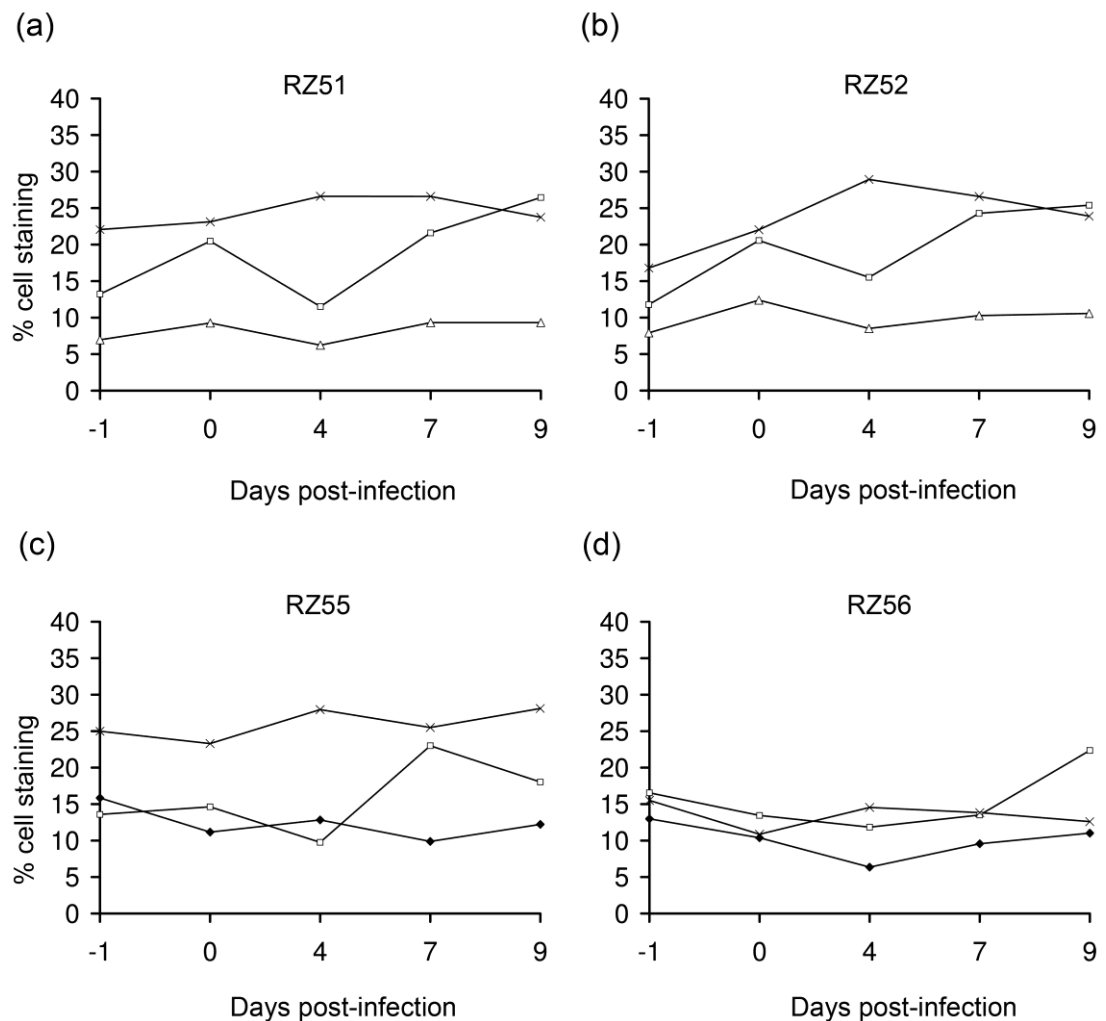


Figure 51. Effect of anti-WC1 and anti-CD8 MAb administration on the percentage of T lymphocyte subpopulation in the peripheral blood not targeted for depletion, measured by flow cytometry.

(a) to (d) Experiment 1 animals, MAbs were administered over three days starting the day before FMDV challenge (RZ51 and RZ52, anti-WC1 MAb. RZ55 and RZ56, anti-CD8 MAb). \diamond = WC1⁺ cells, \square = CD4⁺ cells, \triangle = CD8⁺ cells and \times = CD21⁺ cells. Administering anti-WC1 or anti-CD8 MAbs did not result in non-specific depletion of other cell types.

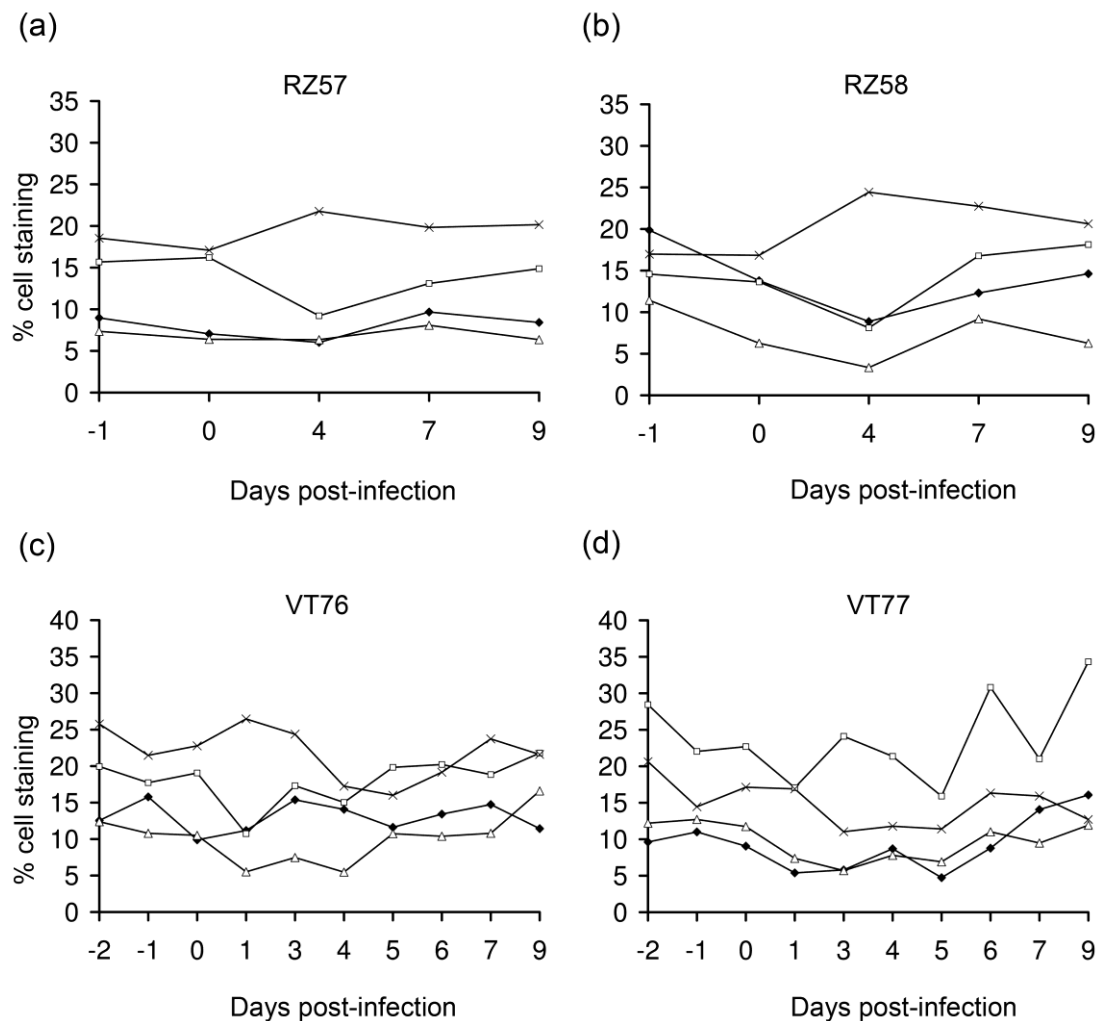


Figure 52. Effect of TRT3 MAb administration on the percentage of T lymphocyte subpopulation in the peripheral blood not targeted for depletion, measured by flow cytometry. a) to (b) Experiment 1 animals, MABs were administered over three days starting the day before FMDV challenge. (c) and (d) Experiment 2 animals, Mabs were administered over four days starting two days before FMDV challenge. ♦ = WC1⁺ cells, □ = CD4⁺ cells, △ = CD8⁺ cells and × = CD21⁺ cells. Administering MAb TRT3 did not result in non-specific depletion. In addition, there were no major changes in the proportions of the lymphocyte subsets during acute infection, although some fluctuation in the percentage representation of each subset was observed during the course of the studies.

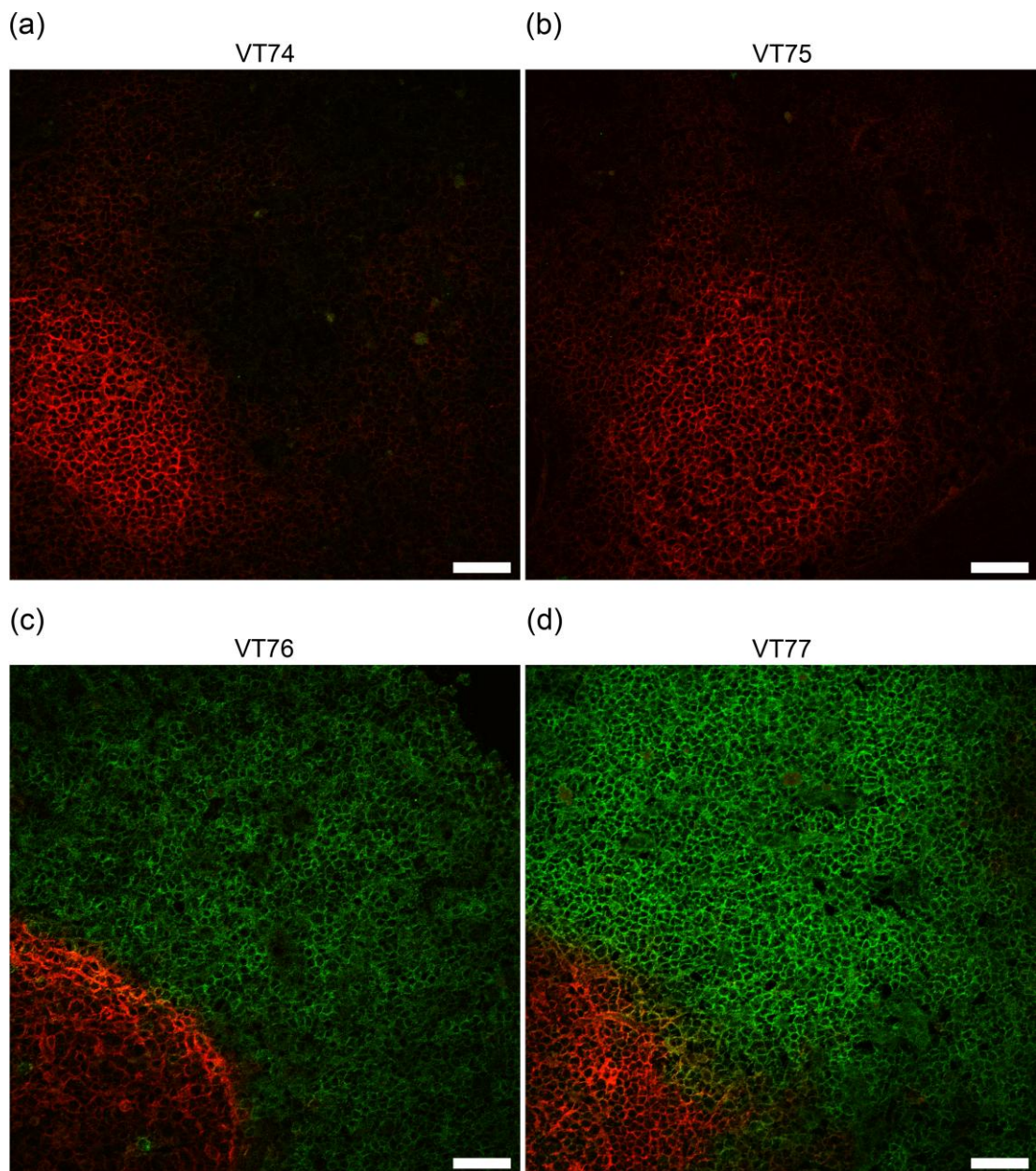


Figure 53. Effect of anti-CD4 MAb injection on the target cell population in lymphoid tissue. (a) to (d) Immunofluorescence confocal microscopy images of prescapular lymph node cortices from experiment 2: anti-CD4 MAb (VT74, VT75), and TRT3 control MAb (VT76, VT77) injected animals biopsied at 5 days post-infection. CD4⁺ lymphocytes stained green (MAb CC30), CD21⁺ cells stained red (MAb CC51). Scale bars represent 40µm.

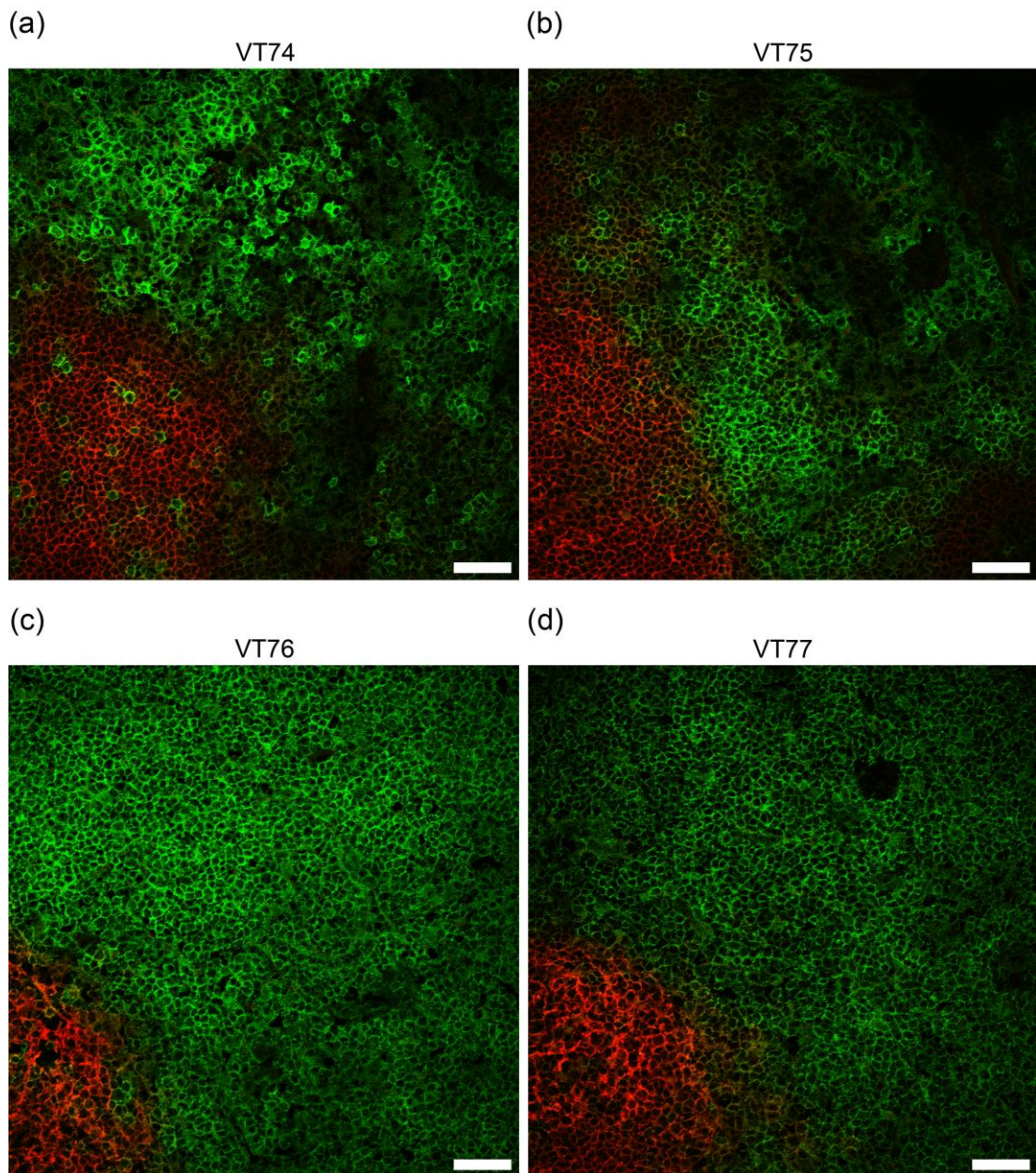


Figure 54. CD3⁺ T cells were readily detectable in cryosections of prescapular lymph nodes biopsied at 5 days post-intradermolingual challenge.

(a) to (d) Immunofluorescence confocal microscopy images of prescapular lymph node cortices from experiment 2: anti-CD4 MAb CC8 and IL-A11 (VT74, VT75), and TRT3 control MAb (VT76, VT77. Anti-turkey rhinotracheitis virus) injected animals. CD3⁺ lymphocytes stained green (anti-CD3 MAb MM1A), CD21⁺ cells stained red (anti-CD21 MAb CC51). Scale bars represent 40µm.

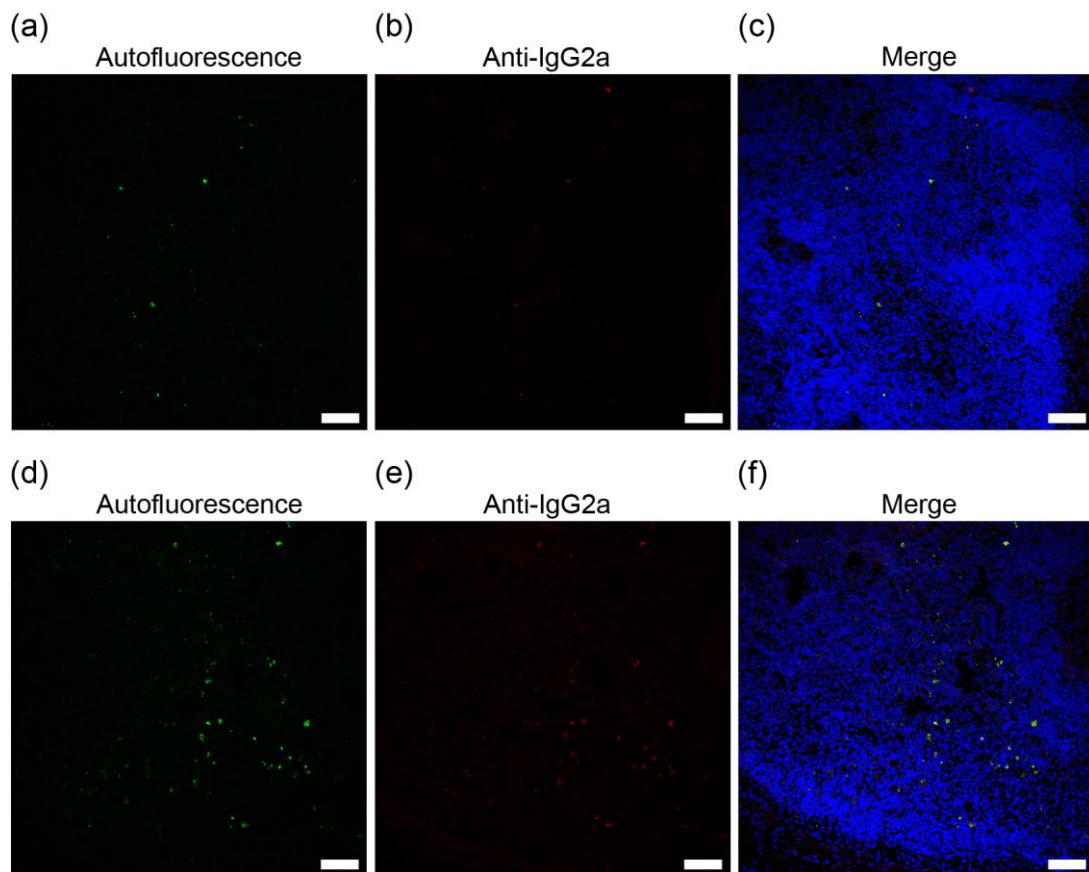


Figure 55. The anti-CD4 MABs used for depletion could not be detected in the prescapular lymph node cryosections harvested at 5 days post-intradermolingual challenge.

Cryosections harvested at 5 days post-intradermolingual challenge from experiment 2: CC8 and IL-A11 (anti-CD4 MABs, IgG2a isotype) treated animals VT74 (a) to (c), and VT75 (d) to (f). (a) and (d) Autofluorescence (green) associated with bovine lymph nodes. (b) and (e) No signal above background detected with anti-IgG2a secondary MAB (red). (c) Merge image of (a) and (b). (f) Merge image of (d) and (e). Nuclei stained blue in merge images (DAPI), scale bars represent 80μm.

3.4.2. Effect of lymphocyte depletion on development of clinical FMD

The clinical scores for all animals following FMDV infection, representing a measure of the induction, severity and resolution of clinical signs, are displayed in Figure 56. All cattle succumbed to disease within 1 to 3 days post-challenge. T-cell depletion had no adverse effect on the onset, magnitude or resolution of clinical signs following infection. Milder clinical scores were recorded for one of the CD4 depleted animals (RZ53), however, it is unlikely that this observation is significant considering the spectrum of clinical signs seen after FMDV challenge (Alexandersen *et al.*, 2003b).

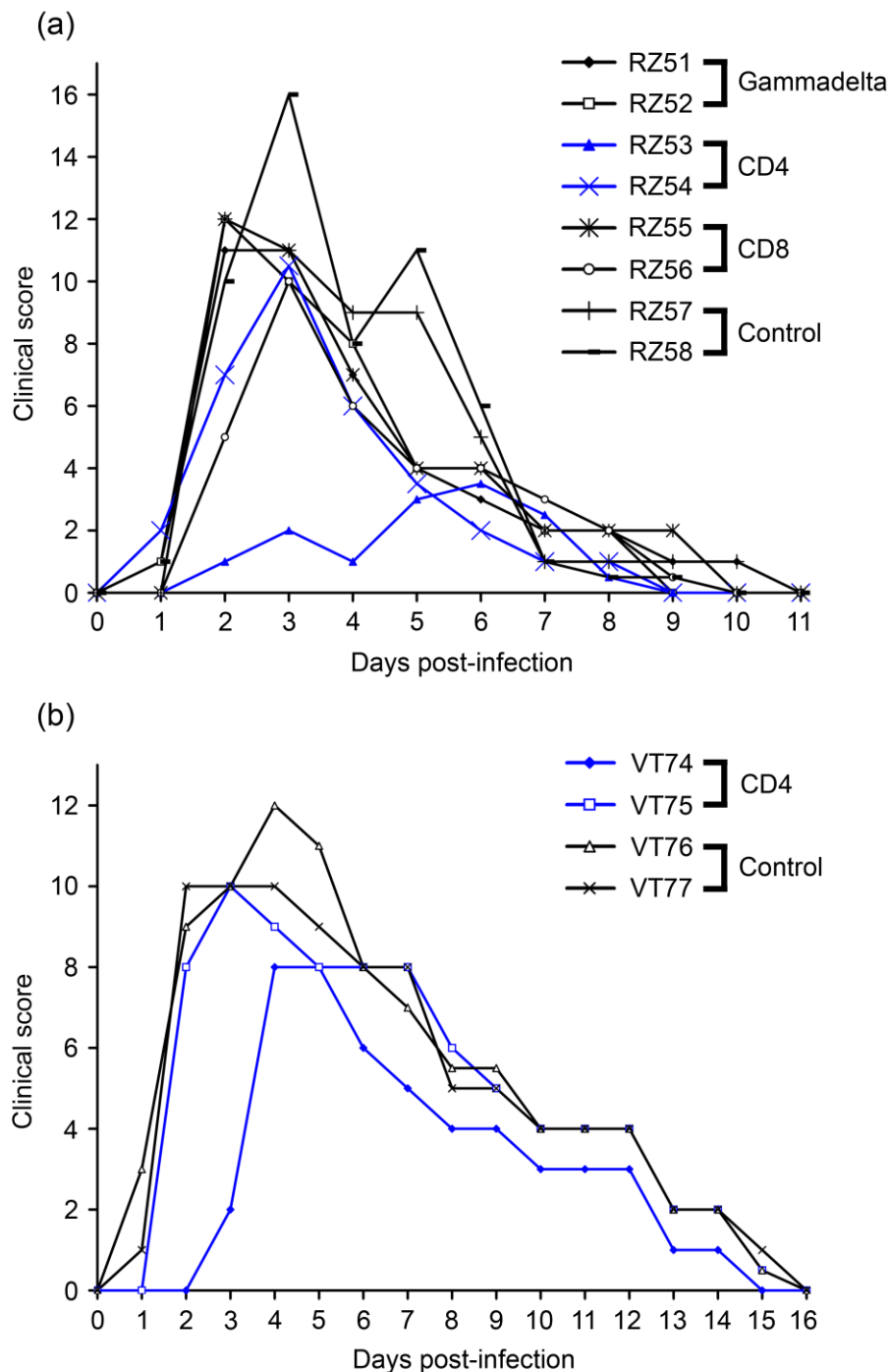


Figure 56. Effect of lymphocyte depletion on development of clinical FMD.

The clinical scores, consisting of rectal temperature and clinical signs of FMD (Table 6), are displayed for experiment 1 animals (a) and experiment 2 animals (b). The data related to the anti-CD4 MAb treated animals are highlighted in blue. T-cell depletion had no adverse effect on the onset, magnitude or resolution of clinical signs following infection.

3.4.3. Effect of lymphocyte depletion on viral clearance

All animals were confirmed viraemic 24 hours post-infection by virus isolation and quantitative rRT-PCR. The results of daily quantitative measures of viral genome in serum determined by rRT-PCR are presented in Figure 57. High levels of viral genome were detected in serum collected on days 1, 2 and 3 in all groups of animals and subsequently declined in all groups. Viral genome was no longer detectable in all except two animals, one control and one CD8⁺ T-cell-depleted animal, by day 7 after infection. No serum samples were collected on day 8, but samples from the two remaining positive animals were negative for viral genome on day 9. There was no significant difference in the peak level of viraemia, as measured by rRT-PCR, between any of the different MAb-treated groups ($P = 0.297$, ANOVA. General linear model). By inspection, one cannot rule out a minor influence of WC1⁺ cell depletion on the duration of viraemia as measured by rRT-PCR (Figure 57), although it was not possible to assess the significance of this observation due to the small group size. Live virus was isolated from serum samples of animals treated with anti-CD4 and anti-CD8 MAb up to 4 days post-infection, and from animals treated with anti-WC1 and control MAb up to 3 days post-infection. No live virus or viral genome was detected in probang samples at post-mortem by virus isolation and rRT-PCR. FMDV capsid protein was detected by immunofluorescence confocal microscopy in germinal centres of mandibular lymph nodes harvested from all animals at post-mortem (day 30 for animals in experiment 1 and on day 29 for animals in experiment 2) with data from the anti-CD4 MAb treated animals presented in Figure 58 and Figure 59.

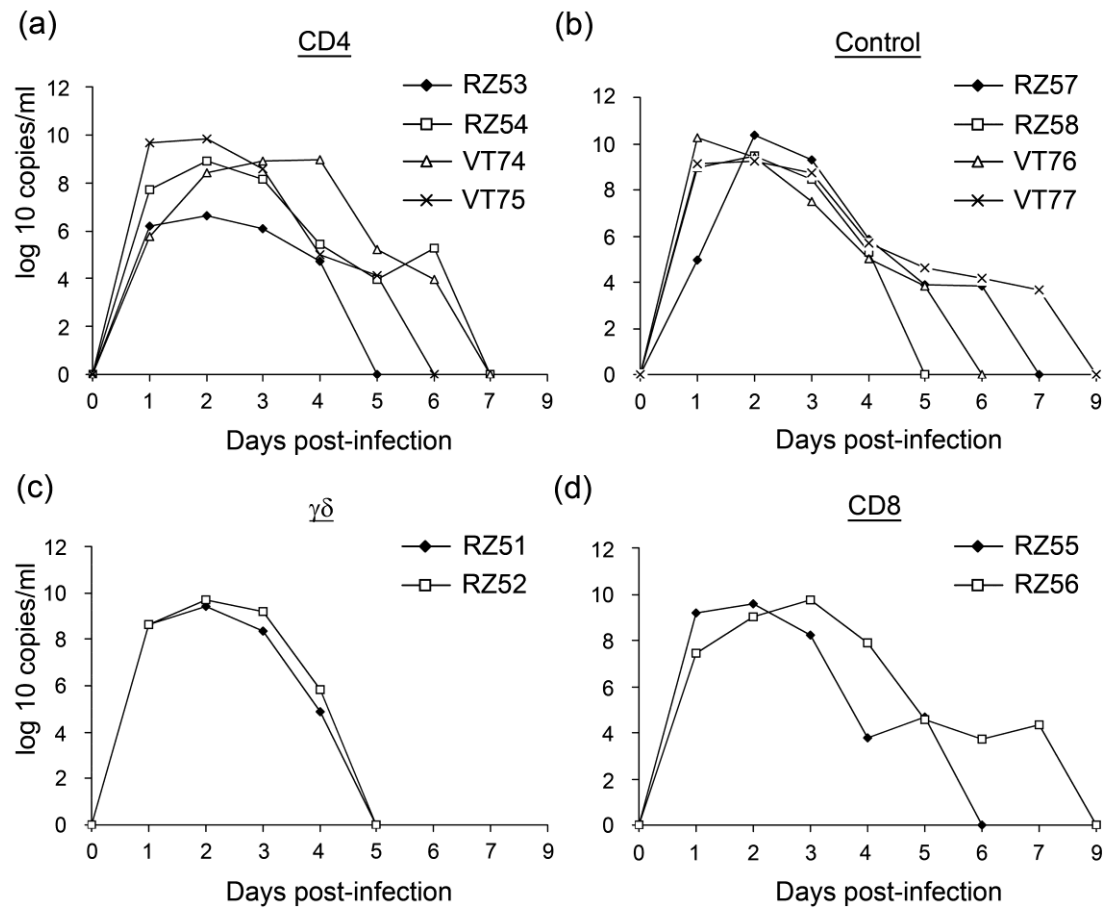


Figure 57. Effect of lymphocyte depletion on viraemia.

Viral genome was detected by rRT-PCR in serum samples collected from day 0 to 7 and day 9 post-infection. Genome copies per mL serum are displayed in panel (a) for anti-CD4 MAb treated and (b) TRT3 control MAb treated animals from both experiments, (c) anti-WC1 MAb treated and (d) anti-CD8 MAb treated animals from experiment 1.

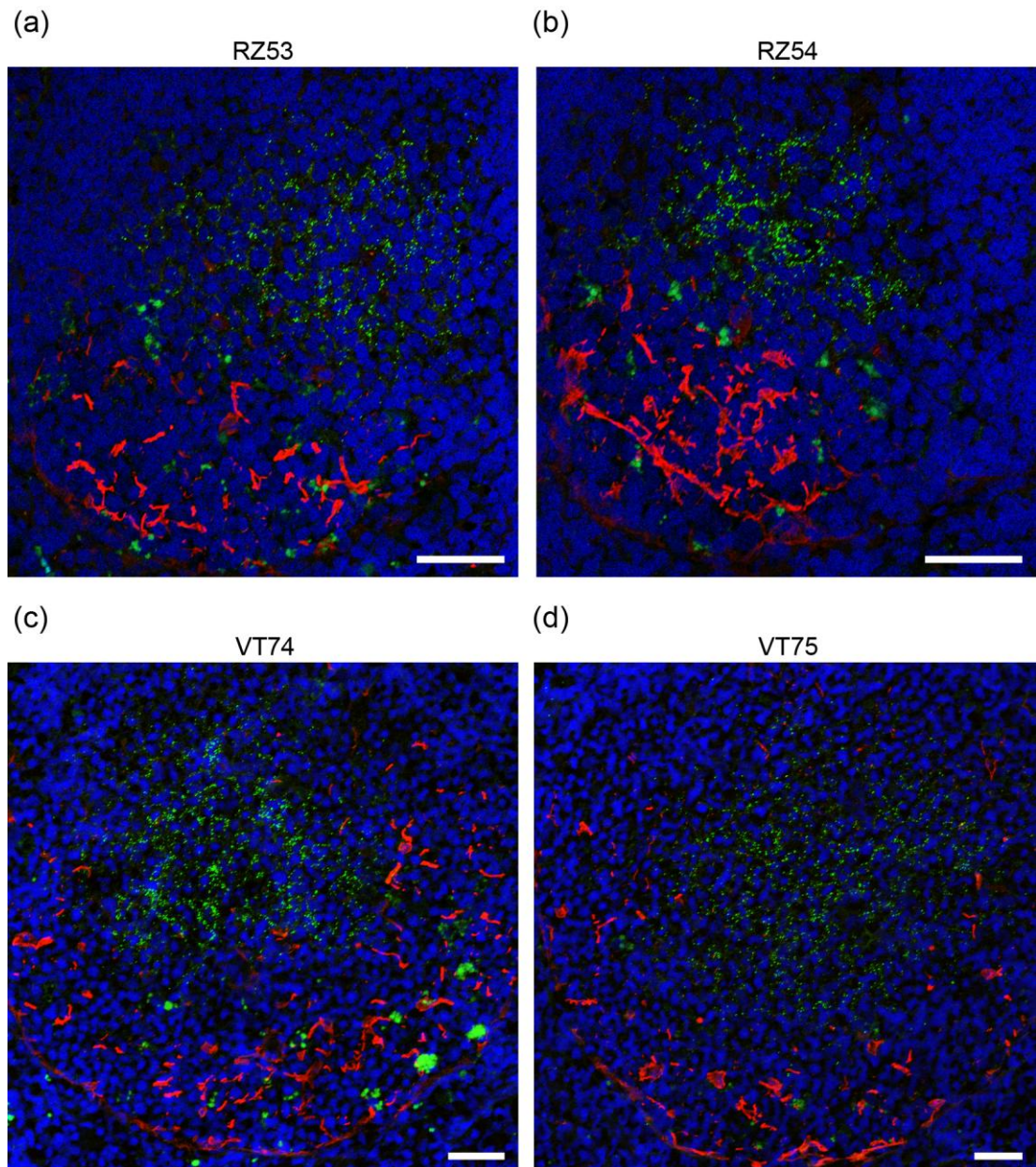


Figure 58. FMDV capsid detected in the light zone of mandibular lymph node germinal centres at post-mortem.

(a) to (d) Mandibular lymph node cryosections harvested at post-mortem from anti-CD4 MAb treated animals on day 30 for experiment 1 (RZ53, RZ54. Anti-CD4 MAb CC8) and on day 29 for experiment 2 (VT74, VT75. Anti-CD4 MAbs CC8 and IL-A11). Panels are merge images of fibrinogen, associated with dark zone FDCs, stained red with MAb D46, FMDV capsid stained green with MAb IB11 and nuclei stained blue (DAPI). Scale bars represent 50µm.

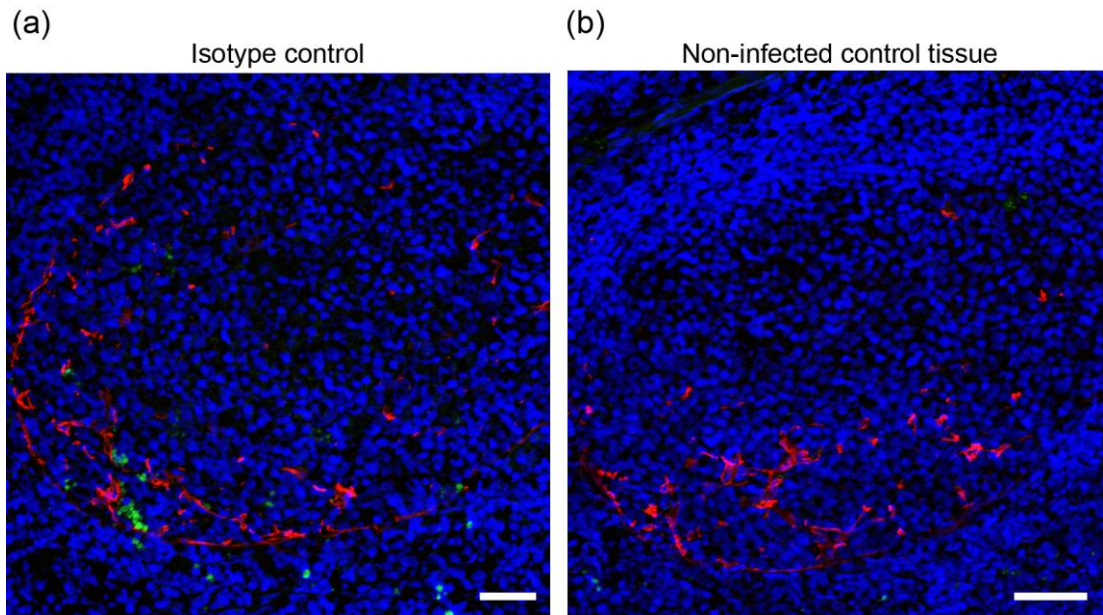


Figure 59. No signal detected in the light zone of control mandibular lymph node germinal centre cryosections.

(a) Mandibular lymph node cryosection harvested at post-mortem on day 29 from experiment 2, anti-CD4 MAb (anti-CD4 MAbs CC8 and IL-A11) treated animal VT74. Fibrinogen, associated with dark zone FDCs, stained red with MAb D46. No signal could be detected with isotype control MAb TRT1 (anti-turkey rhinotracheitis virus) stained green. (b) Mandibular lymph node from a non-infected control animal. Fibrinogen stained red (anti-fibrinogen MAb D46). No signal detected with anti-FMDV capsid MAb IB11 (green). Nuclei stained blue (DAPI). Scale bars represent 50µm.

3.4.4. Effect of lymphocyte depletion on virus neutralising antibody

The results of virus neutralising antibody assays of serum samples are displayed in Figure 60. Titres of ≥ 45 (considered positive) were attained by 5 days post-infection in all 4 control animals, by 4 to 7 days in the animals treated with anti-CD4 MAb and by 5 to 6 days in the animals treated with the anti-WC1 and anti-CD8 MAb. There were no obvious differences in the onset of detectable neutralising antibody in the calves receiving the different antibody treatments. In particular, the onset of detectable neutralising antibody titres post-infection was not significantly different in the calves treated with anti-CD4 antibody and control antibody ($P = 0.11$, Kruskal-Wallis test). The complete data set of virus neutralising antibody titres can be found in Table 8.

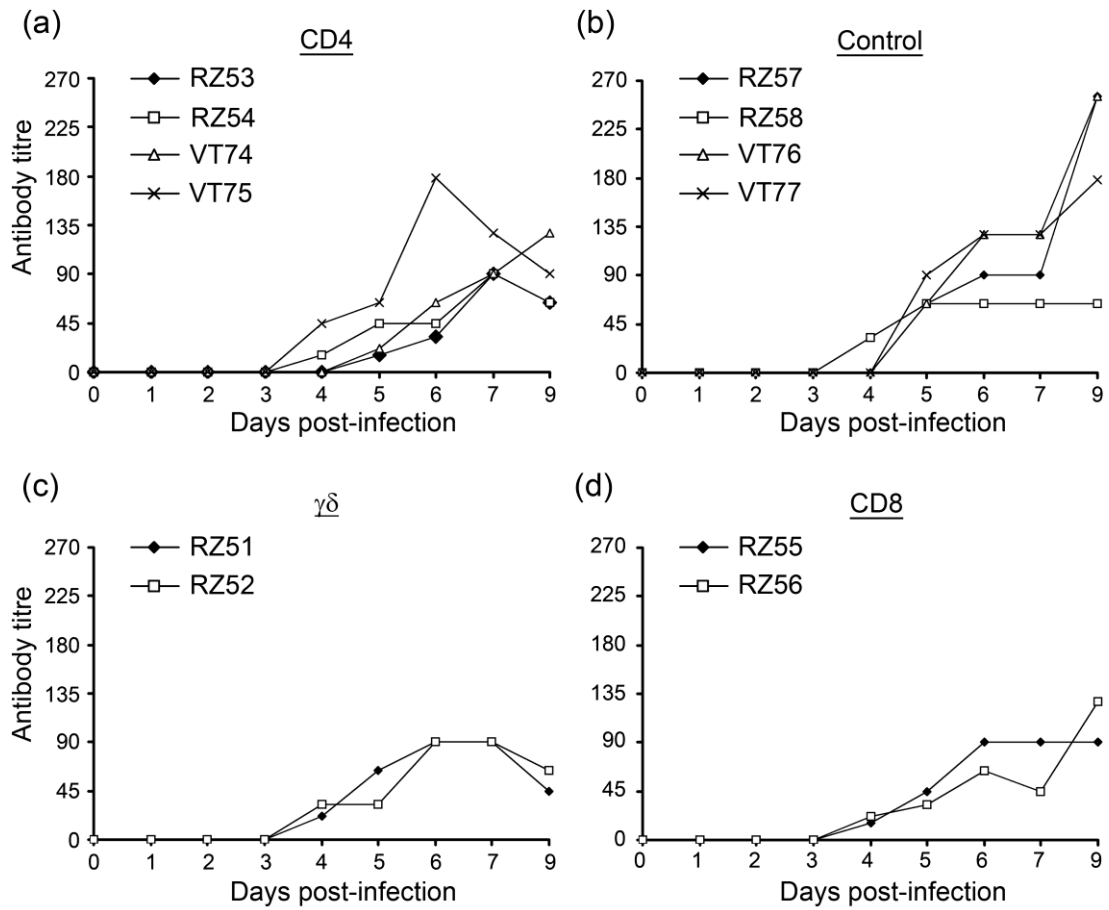


Figure 60. Effect of lymphocyte depletion on virus neutralising antibody.

Virus neutralising antibody titres are displayed in panel (a) for anti-CD4 MAb treated and (b) TRT3 control MAb treated animals from both experiments, (c) anti-WC1 MAb treated and (d) anti-CD8 MAb treated animals from experiment 1. A titre of ≥ 45 is considered positive.

Table 8. Virus neutralising antibody titres of experiment 1 (RZ51 to RZ58) and experiment 2 (VT74 to VT77) animals.

Study day	TRT3				Anti-CD4				Anti-WC1		Anti-CD8	
	RZ57	RZ58	VT76	VT77	RZ53	RZ54	VT74	VT75	RZ51	RZ52	RZ55	RZ56
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	32	0	0	0	16	0	45	22	32	16	22
5	64	64	64	90	16	45	22	64	64	32	45	32
6	90	64	128	128	32	45	64	178	90	90	90	64
7	90	64	128	128	90	90	90	128	90	90	90	45
9	256	64	256	178	64	64	128	90	45	64	90	128
13	256	178	128	355	64	178	90	178	128	178	128	178
16	355	178	355	1024	90	178	512	1024	256	512	256	256
21	355	355	708	708	90	355	512	1024	256	355	512	256
23	512	512	708	1024	178	708	512	708	256	512	512	512
27	512	355	708	1024	178	355	708	708	512	256	178	355
29/30	128	128	1024	1413	256	256	1024	708	355	512	256	355

3.4.5. Effect of lymphocyte depletion on the antibody response to FMDV non-structural proteins

Serum samples collected at 3 to 6 day intervals, from days 0 to day 29 (experiment 2) or 30 (experiment 1) post-infection, were analysed for the presence of antibodies against the FMDV non-structural protein 3ABC. The kinetics of the antibody response to 3ABC in animals receiving anti-CD8 or anti-WC1 MAb were similar to that of the control animals, with antibody initially detected on days 6 to 16 and maximum titres were detected on day 29 or 30. In contrast, three out of the four anti-CD4 MAb treated animals had no detectable antibodies against 3ABC throughout the 29 to 30 days and the fourth animal (VT75) remained negative until day 29. Titres of anti-3ABC antibodies in serum samples obtained at the time of post-mortem (days 29 or 30) are shown in Figure 61. These results indicate that depletion of CD4⁺ T cells during the phase of acute FMDV replication ablates the antibody response to non-structural viral proteins.

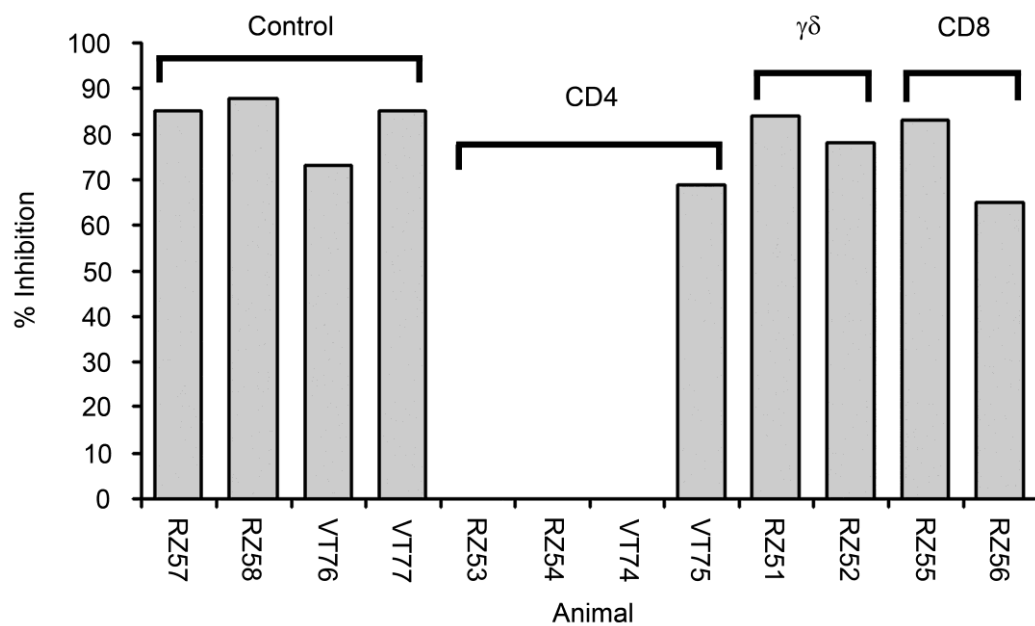


Figure 61. Effect of lymphocyte depletion on the response to FMDV non-structural protein 3ABC.

By day 29/30 post-infection, three anti-CD4 MAb treated animals had no detectable antibody response to the FMDV non-structural protein 3ABC. Samples were considered positive if the percentage inhibition was ≥ 50 (Sorensen *et al.*, 1998). Control: TRT3 MAb treated animals from both experiments. CD4: anti-CD4 MAb treated animals from both experiments. VT75: an experiment 2 higher antibody dose animal. WC1: anti-WC1 MAb treated and CD8: anti-CD8 MAb treated experiment 1 animals.

3.4.6. Effect of lymphocyte depletion on the isotype of FMDV-specific antibody responses

Serum samples collected daily during the first 7 days of infection and at 2 to 5 day intervals up to day 29 (experiment 2) or 30 (experiment 1) post-infection were analysed using an ELISA with reagents specific for bovine IgM, IgG1 and IgG2, to determine the kinetics of the various isotypes generated by the FMDV-specific antibody response. Comparison of the kinetics of antibody titres over time by parallel curve analysis (see section 3.3.13) did not reveal any statistically significant differences between the responses of animals in MAb-treated groups and those in the control MAb-treated groups (*P* values of 0.44, 0.43 and 0.61 for IgM, IgG1 and IgG2 respectively). Examples of the profiles of the FMDV-specific antibody responses of the 3 anti-CD4 MAb treated animals with no detectable antibody response to FMDV 3ABC and a control animal are displayed in Figure 62. IgG antibody isotypes were detected 5 to 7 days after infection indicating rapid isotype switching in all animals. Indeed, in some cases specific IgG2 antibodies were detected earlier than IgM antibodies. Antibody isotype class switching occurred during the phase of CD4⁺ T-cell depletion in animals that received anti-CD4 MAb.

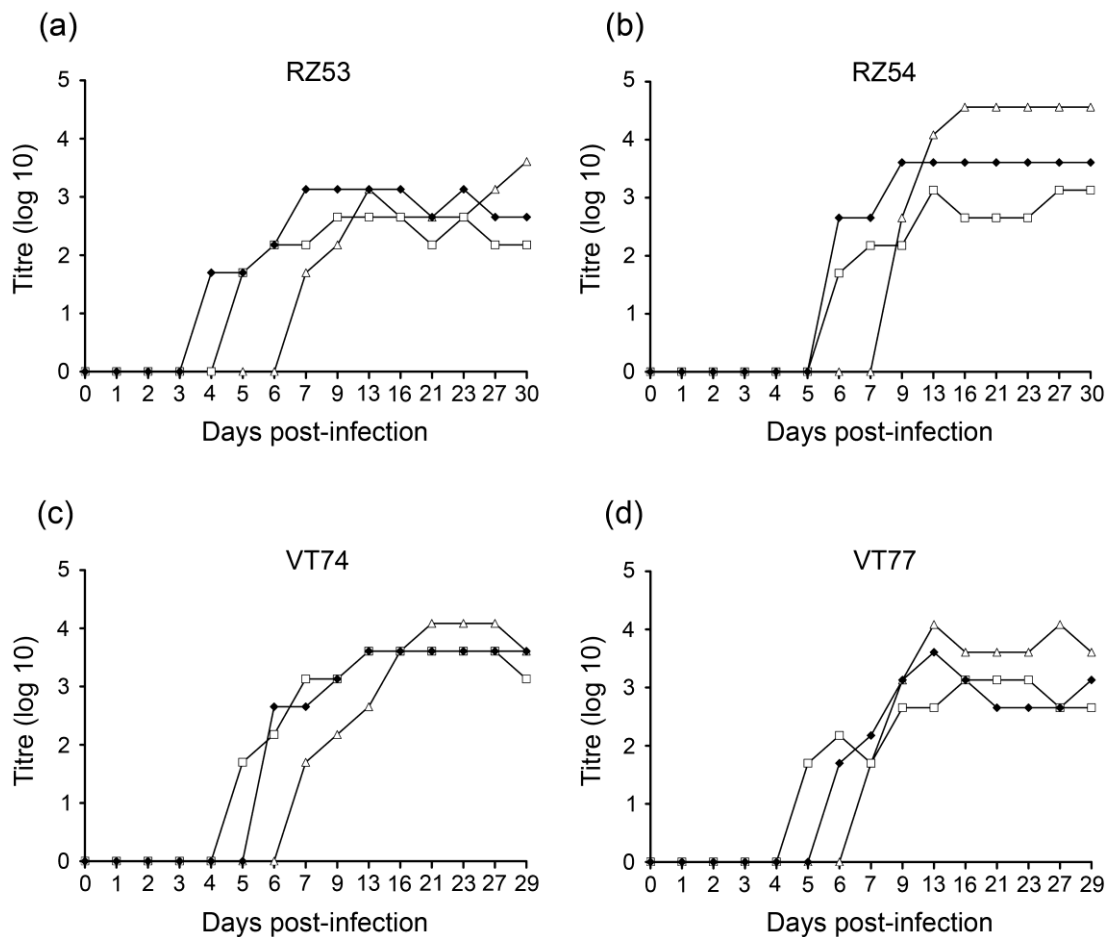


Figure 62. Effect of lymphocyte depletion on the isotype of FMDV-specific antibody responses. Examples of the FMDV-specific antibody isotype profiles are displayed in panel (a) and (b), for experiment 1 and (c), for experiment 2 anti-CD4 MAb treated animals with no detectable antibody response to FMDV 3ABC. (d) TRT3 control MAb treated animal from experiment 2. IgG1 = \triangle , IgG2 = \square , IgM = \blacklozenge . Efficient antibody isotype class switching occurred during the period of CD4⁺ T-cell depletion.

3.4.7. Effect of lymphocyte depletion on the antibody response to G-H loop peptides

Serum samples from animals receiving anti-CD4 MAbs and those receiving the control MAbs in both experiments were examined using an indirect ELISA for the presence of IgG antibodies to O UKG 34/2001 and O1BFS VP1₁₃₅₋₁₅₆ peptide, which represent a superficial loop exposed on the surface of the viral capsid. No antibodies directed against the peptides were detected pre-challenge. Titres of antibody specific for the peptides detected prior to the re-appearance of circulating CD4⁺ T cells following depletion (day 7 post-infection for experiment 1, day 9 for experiment 2 - Figure 49), and following CD4⁺ T cell repopulation (day 16) are displayed in Figure 63. By the end of the period of CD4⁺ cell depletion, the 4 infected control animals all showed detectable antibody responses to the O UKG G-H loop peptide at day 7 (experiment 1) or day 9 (experiment 2). In contrast, antibody was undetectable in two of the CD4 T-cell-depleted animals and present at a very low titre in the other 2 depleted animals at these time points. By day 16, the titre of antibody in 3 of the depleted animals was still less than in the controls (however, due to the small numbers of animals it was not possible to determine if the difference was statistically significant). These findings were corroborated by the data for the O1BFS peptide indicating that the antibody response to the G-H loop was inhibited by CD4⁺ T-cell depletion.

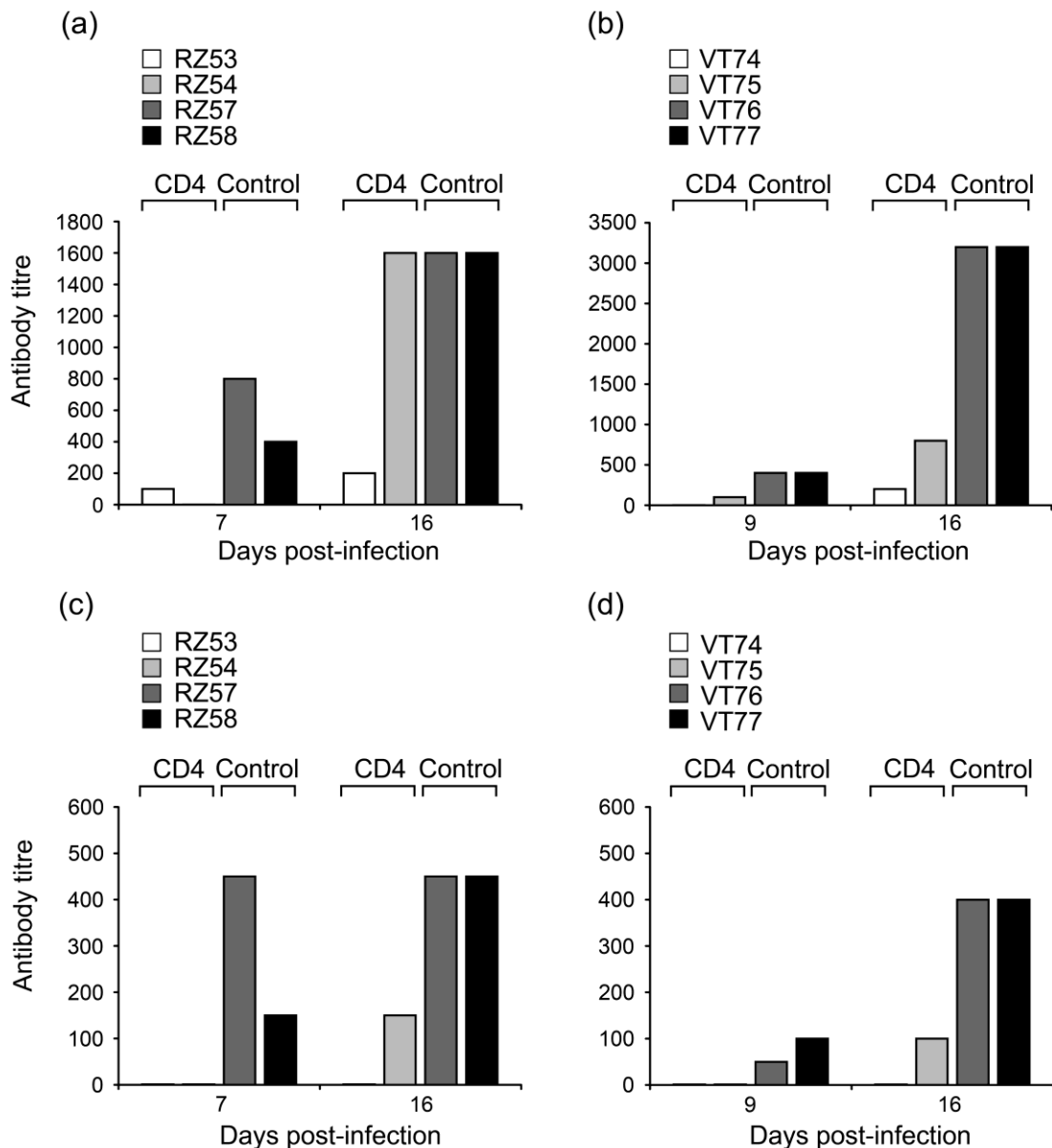


Figure 63. Effect of lymphocyte depletion on the antibody response to G-H loop peptides. No antibodies directed against the peptides were detected pre-challenge. (a) the IgG antibody response of experiment 1, CD4 depleted animals to FMDV O UKG 34/2001 VP1₁₃₅₋₁₅₆ G-H loop peptide was absent or substantially less than that of the control animals by day 7 post-infection. By day 16, a stage when CD4 cells were repopulating, the levels of antibody in the CD4 depleted animals were less than or equal to that of the controls. (b) The antibody response of experiment 2 CD4 depleted animals was similarly absent or substantially less than that of the control animals by day 9 post-infection. Although CD4 cells were repopulating by day 16 post-infection, the response of the experiment 2, CD4 depleted animals was still substantially less than that of the controls. (c) and (d) These findings were corroborated by the data for the O1BFS peptide performed using 2 µg/mL peptide as previously described (Fowler *et al.*, 2008). These results indicate that the antibody response to the G-H loop was inhibited by CD4⁺ T-cell depletion.

3.5. Discussion

These data confirm that depletion of CD4⁺ lymphocytes from the blood circulation and superficial lymph nodes can be achieved in cattle by administering sufficient quantities of specific mouse MAbs. The application of different CD4 depletion protocols in calves during the early stages of infection with FMDV was found to result in similar, substantial ablation of IgG antibody responses to non-structural viral proteins but had little impact on the antibody responses to sites on the surface of the virus particles that induce neutralising antibodies. Depletion of CD4 T cells also had no significant effect on the course of viraemia or the clinical severity of disease associated with FMDV infection. Milder clinical scores were recorded for one of the CD4 depleted animals (RZ53), however, it is unlikely that this observation is significant considering the spectrum of clinical signs seen after FMDV challenge (Alexandersen *et al.*, 2003b). There was no CD4 T-cell depletion in control animals following FMDV infection which contrasts with the significant lymphopenia reported in swine following FMDV infection (Bautista *et al.*, 2003).

Although administration of anti-WC1 antibody was also found to result in profound depletion of circulating WC1⁺ $\gamma\delta$ T cells, such depletion did not have any measurable effect on the course of infection with FMDV or specific antibody responses to the virus. The role of these cells in protection against infectious agents in ruminants is unclear. Epithelial tissue contains large numbers of $\gamma\delta$ T cells (Howard *et al.*, 1989) and these cells have been proposed to play a role in controlling intracellular infections, promoting a Th1-biased immune response (Pollock and Welsh, 2002) and

non-MHC restricted NK-like cytotoxicity (Brown *et al.*, 1994, Daubenberger *et al.*, 1999). Previous reports of WC1⁺ T-cell depletion studies in cattle have shown an enhanced antibody response to non-replicating antigen, and an enhanced PBMC proliferative response to non-specific mitogens in animals depleted of this population (Howard *et al.*, 1989). These results were supported further by the detection of enhanced local and systemic IgM and IgA antibody responses following respiratory syncytial virus infection in WC1⁺ depleted calves (Taylor *et al.*, 1995). The enhanced antibody responses reported in these previous studies may be as a result of higher levels of antigen at the early stages of infection (Taylor *et al.*, 1995) or as a result of greater Th2-bias in the immune response suggested by higher levels of IL-4, lower levels of IFN- γ and reduced levels of IgG2 antibody (Kennedy *et al.*, 2002). By inspection, one cannot rule out a minor influence of WC1⁺ cell depletion on the duration of viraemia as measured by rRT-PCR (Figure 57), although it was not possible to assess the significance of this observation due to the small group size. Overall, our findings suggest that WC1⁺ $\gamma\delta$ T cells do not play a major role in the resolution of clinical signs and control of viraemia after acute FMDV infection in cattle.

Application of a similar protocol to deplete CD8⁺ T cells was less successful, resulting in only partial depletion of the circulating population, which had no discernible effect on the response to FMDV. This result is consistent with previous evidence that MAb-mediated depletion of bovine CD8⁺ T cells is more difficult to achieve than for other T-cell subsets (Naessens *et al.*, 1998, Oldham *et al.*, 1993, Taylor *et al.*, 1995, Villarreal-Ramos *et al.*, 2003). Therefore, it was not possible to

conclusively evaluate the influence of CD8⁺ T cells on the course of infection with FMDV or early responses to the virus. However, partial depletion of CD8⁺ T cells did not affect the resolution of acute FMDV infection.

CD4⁺ T-cell depletion did not influence the development of FMDV neutralising antibody. Antiviral antibody responses may be classified as T-D or T-I based on the requirement for CD4⁺ T cell help for antibody production. T-I type I antigens are mitogenic agents, for example lipopolysaccharides, that activate Toll-like receptors to elicit polyclonal B cell activation (Obukhanych and Nussenzweig, 2006). Type II T-I antigens are complex structures, typically rigid two dimensional arrays comprising repeating epitopes displayed at 5 to 10nm intervals, that engage and cross-link the immunoglobulin receptors on the surface of B cells generating strong activation signals. These stimulatory activities result in antibody production in the absence of specific T cell help but may depend upon accessory signals from antigen presenting cells or T cells for B-cell activation (Bachmann and Zinkernagel, 1997, Hangartner *et al.*, 2006, Mond *et al.*, 1995, Morrissey *et al.*, 1981). Some viral capsids fall into this category. However, non-oligomerised viral proteins released from dying cells or disrupted virus particles generally act as T-D antigens.

The T-dependency of antibody responses of cattle to a number of defined antigens and viral pathogens has been confirmed in several previous studies (Howard *et al.*, 1992, Naessens *et al.*, 1998, Taylor *et al.*, 1995). CD4⁺ lymphocyte depletion with MAbs doses as low as 0.3mg/kg has been shown to result in a significant reduction in the antibody response of calves to human red blood cells and ovalbumin (Howard *et*

al., 1989, Naessens *et al.*, 1998). The same dose of MAb administered to calves subsequently infected with respiratory syncytial virus resulted in a marked suppressive effect on the antibody response and increased viral pathology (Naessens *et al.*, 1998, Taylor *et al.*, 1995). Similar results have been reported after infection with non-cytopathic bovine viral diarrhoea virus, where incomplete circulating CD4⁺-lymphocyte depletion resulted in a delayed antibody response and longer duration and higher titre of circulating virus (Howard *et al.*, 1992). Furthermore, depletion of CD4⁺ lymphocytes in cattle previously vaccinated with commercial FMDV vaccine has been shown to ablate T-cell-proliferative responses to FMDV antigen, indicating depletion of memory T cells (Naessens *et al.*, 1998). While Naessens *et al.* depleted blood and splenic CD4⁺ cells with 0.2mg/kg MAb, they needed 2mg/kg to deplete CD4⁺ cells from peripheral lymph nodes. It is therefore likely that 2.58mg/kg MAb was effective at depleting the cells from peripheral lymph nodes, confirmed in our analyses on the prescapular lymph nodes. Moreover, the present work clearly demonstrated that such depletion had a strong influence on the anti-FMDV immune response, but this was prejudiced dependent on the antigenic determinants against which the humoral response was mounted. A particularly significant feature of the present study was the finding that CD4⁺ T-cell depletion resulted in ablation of antibody responses to non-structural proteins in 3 of the 4 animals examined, while leaving intact the antibody responses to sites on the surface of the viral capsid. This is consistent with the notion that antibody responses to these antigenic components are T-D and T-I respectively. The development of a delayed antibody response to the non-structural proteins in one of the CD4 depleted calves

may be the result of low level replicating virus still being present in this animal when CD4⁺ T-cell function was restored.

Our findings are consistent with published results using the FMDV murine experimental model, where the protective immune response was shown to be T-I (Borca *et al.*, 1986, Lopez *et al.*, 1990). These investigators showed that after FMDV challenge, the curves of viraemia and neutralising antibody responses in the athymic mice were not significantly different to those of the normal control mice (Borca *et al.*, 1986). In addition, the athymic mice were protected from re-challenge 240 days post-infection, indicating that FMDV induces a prolonged, T-I immune memory response in mice (Lopez *et al.*, 1990). Early T-I protection and production of antibody has been described for a number of other cytopathic viruses, including VSV and influenza virus infection in mice (Fehr *et al.*, 1996, Lee *et al.*, 2005). A number of other picornaviruses have also been shown to act as T-I antigens (Bachmann and Zinkernagel, 1996). The T-I nature of these viral antigens is thought to be a result of their rigid, highly repetitive and highly organised structure (Bachmann and Zinkernagel, 1997). Also, the magnitude of the T-I immune response and augmentation of antibody isotype class switching has been shown to correlate with the degree of antigen organisation and the dose of antigen reaching the secondary lymphoid organs (Bachmann and Zinkernagel, 1996, Maloy *et al.*, 1998, Ochsenbein *et al.*, 2000a, Zinkernagel, 2000). One of the key protective mechanisms to prevent the dissemination in the host of acute cytopathic viruses is the rapid induction of neutralising antibodies (Bachmann and Zinkernagel, 1997). It has also been proposed that the surface antigenic structure of acute cytopathic viruses has evolved to

stimulate early T-I antibody responses, in order to limit the extent of viral infection and avoid rapid death of the host. Conversely, B-cell responses may have evolved to deal with such threats. The dynamics of infection with FMDV in cattle is consistent with the model described above, with infection being rapidly controlled and animals usually showing clinical signs only for a few days. Clearly, T-D antibody responses are also stimulated by these acute cytopathic viruses, and are likely to be responsible for the production of affinity matured IgG-isotype antibodies and long term memory (Hangartner *et al.*, 2006).

Although FMDV shares structural features with other picornaviruses, there is one unique feature that distinguishes aphthoviruses including FMDV from other picornaviruses; the absence of a canyon or pit which places the integrin cell attachment site in the protruding, fully exposed, highly disordered and mobile immunogenic G-H loop of VP1 (Acharya *et al.*, 1989). Studies with virus-specific MAbs, coupled with structural analyses of FMDV particles, have identified 5 antigenic sites on the FMDV capsid, including the G-H loop, which are involved in virus neutralisation (Crowther *et al.*, 1993). The G-H loop is considered highly immunogenic, and immunisation of cattle with synthetic peptides representing the loop has been shown to induce neutralising antibody and in some cases protection against viral challenge (Taboga *et al.*, 1997). However, recent data describing VP1 G-H loop-substituted chimeric vaccines indicates that the G-H loop may not be required for producing a strong neutralising antibody response or a protective immune response following vaccination in cattle (Fowler *et al.*, 2008). In the present study, although CD4⁺ T-cell depletion had no discernible effect on the overall

neutralising antibody response, it substantially inhibited the IgG antibody response against the G-H loop peptide. The neutralising antibody in these animals was presumably directed against the other sites on the viral capsid. Our data suggest that the high degree of mobility of the G-H loop may result in it being less effective as a T-I type II antigen in comparison with the other antigenic sites, which have a more stable conformational structure. Antibodies directed against the G-H loop were detected in all CD4⁺ T-cell-depleted cattle after the phase of depletion, albeit at lower levels than the infected control animals. Although circulating virus was no longer detectable at this time, the detection of FMDV capsid antigen in mandibular lymph node germinal centres at post-mortem indicates that there remained a source of antigen for induction of G-H loop-specific antibody when CD4⁺ T cell function was restored.

The induction of IgG after FMDV immunisation has been shown to be T-D in a murine experimental model (Collen *et al.*, 1989). These results have been confirmed *in vitro* in a mouse model, in which FMDV-infected DCs could directly stimulate B lymphocytes to secrete FMDV-specific IgM, but T-cell help was required to induce class switching towards IgG (Ostrowski *et al.*, 2007). Comparison of the kinetics of the FMDV-specific antibody response of experiment 1 and 2 animals over time did not reveal any statistically significant differences between the depleted groups and the control MAb-treated groups. Specific serum IgM was detected in these animals from 4 days post-infection and specific IgG1 and IgG2 from 5 days post-infection, consistent with reports by other investigators (Collen, 1994, Doel, 2005, Salt *et al.*, 1996a). Our results show that *in vivo*, in a natural ruminant host, FMDV infection

can not only induce a specific and rapid IgM response but also efficient and rapid isotype class switching in the absence of CD4⁺ T cells. The ability of T-I viral antigens to induce efficient class switching in the absence of T cell help is thought to be related to the repetitiveness of the viral antigens (Bachmann and Zinkernagel, 1997) and the formation of antigen-specific germinal centres by a T-I process in the absence of T cell-derived CD40-ligand (Gaspal *et al.*, 2006). T-I B cell proliferation and isotype class switching in mice following exposure to Type II T-I antigens has been shown to be dependent on an intact follicular dendritic cell network and signalling through CD40 on the surface of B cells and FDCs. The signalling through CD40 is dependent on complement, specifically through C4b binding protein in the absence of the T-cell derived CD40-ligand (CD154) (Brodeur *et al.*, 2003, Gaspal *et al.*, 2006, Ochsenbein *et al.*, 1999b, Schriever *et al.*, 1989, Szomolanyi-Tsuda *et al.*, 2001). We have shown previously in cattle that FMDV localises to germinal centres as early as 3 to 4 days post-challenge (Juleff *et al.*, 2008), a process that may provide the signals required for T-I isotype class switching and an early FMDV-specific IgG response (Gaspal *et al.*, 2006, Ochsenbein *et al.*, 2000a, Tew *et al.*, 2001). The TNF family ligands BAFF and APRIL have also been shown to contribute to CD154-independent antibody isotype switching, germinal centre maintenance and T-I antibody responses (Schneider, 2005). In addition to these potential mechanisms in the CD4⁺ T-cell-depleted cattle exposed to FMDV, IFN γ produced by $\gamma\delta$ T cells (Maloy *et al.*, 1998), NK cells (Koh and Yuan, 1997, Szomolanyi-Tsuda *et al.*, 2001) or activated B cells (Pang *et al.*, 1992, Yoshimoto *et al.*, 1997) may also provide alternative but less efficient support for CD154/CD4⁺ T-I isotype switching by acting

directly on B cells potentially in the absence of specific germinal centre formation (Snapper *et al.*, 1992).

In conclusion, the results of this study indicate that functional CD4⁺ T cells are not required, either to provide help for antibody production or as antiviral effector cells, for effective control of primary infection with FMDV in cattle. Isotype switching of the antibody response was also found to be independent of CD4⁺ T cells. The current studies do not identify whether CD4⁺ T cells play a role in the development or duration of a memory response or contribute to the efficacy of immunity to subsequent viral challenge. Further studies are required to address these questions, possibly using similar depletion protocols in vaccinated animals.

A number of molecular approaches to FMD vaccine development have been followed since the mid-1970s, including the use of viral subunit proteins, protein fragments and peptides, isolated from viral particles or produced in bacteria, baculovirus and transgenic plants or as synthetic peptides (Brown, 1999, Grubman and Mason, 2002, Taboga *et al.*, 1997). A general problem with most subunit vaccines is that they do not elicit a protective immune response comparable with that induced by live virus or killed whole virus vaccines (Taboga *et al.*, 1997). Peptide vaccines based on the G-H loop of VP1 (Wang *et al.*, 2002) do not appear to fully mimic the conformation of the native B-cell epitopes and stimulate limited antibody of rather narrow specificity which can be enhanced by the addition of T-cell epitopes or multiple antigenic sites, but still do not afford adequate protection (Cubillos *et al.*, 2008, Francis *et al.*, 1987, McCullough *et al.*, 1992, Taboga *et al.*, 1997). In contrast,

studies of responses to traditional FMDV vaccines, which utilise intact inactivated virus, have shown that they stimulate rapid antibody responses that can provide protection against disease within 4 to 5 days. The results of the current study, together with other findings, indicate that preservation of the complex three-dimensional structure of the FMDV capsid is critical for inducing rapid and effective antibody responses. This is consistent with current thinking on the development of safer and more effective vaccines based on the use of empty viral capsids produced using recombinant DNA constructs.

4. Conclusion and future work

FMDV infection in cattle provides an opportunity to study the interactions of a highly cytopathic virus which has evolved and adapted to its natural host. During the studies reported in this thesis, FMDV structural and non-structural proteins were detected in cells in sections of bovine lymph node at early time-points post-infection, indicating that viral replication does occur within lymph node cells *in vivo*. However, only small clusters of infected cells were detected. In addition, in the sections of tissue studied, the clusters were restricted to the mandibular lymph node which receives afferent lymph from the tongue, a site associated with vesicles and a high degree of viral replication. Although the significance of this observation is not entirely clear, these data do support a model of natural FMDV infection in cattle during which viable virus is transported to and able to interact with cells in organised lymphoid tissue, which has important immunological consequences. The presence of intact virus within the organised lymphoid tissue, and hence the highly repetitive and ordered capsid antigen, promotes a rapid and effective immune response leading to early induction of antibody, an essential component of the protective immune response against acute cytopathic virus like FMDV (Bachmann and Zinkernagel, 1997, Zinkernagel, 2000).

Notably, intact FMDV capsid was detected in the light zone of mandibular lymph node germinal centres as early as 3 days post-infection. The complement receptors CD21 and CD35 are expressed in both primary and secondary follicles (Imal and Yamakawa, 1996) and may play an important role to trap complement-containing FMDV-immune-complexes formed rapidly after exposure to the pathogen, as is the

case for HIV (Carroll, 1998, Ho *et al.*, 2007). Human FDCs also express the Fc α / μ R for IgM (Kikuno *et al.*, 2007). IgM is typically the first antibody to be produced during a humoral immune response to viral infections and natural antibodies, although not yet described for FMDV in cattle, are mainly IgM (Ochsenbein *et al.*, 1999a). If Fc α / μ R is expressed on bovine FDCs, this receptor may play an important role in membrane-bound antigen presentation to B cells during the initial stages of FMD (Ochsenbein and Zinkernagel, 2000). Although the mechanisms underlying the rapid localisation of FMDV within the germinal centre light zone at this time point are not clear, the rapid formation of antigen-specific germinal centres, and consequent membrane-bound antigen presentation, is likely to be an important component of the immune response against FMDV, able to induce B-cell proliferation and rapid class switching without the need for CD4⁺ T cell help (Gaspal *et al.*, 2006). Clearly, these early events which are capable of efficiently activating the immune system are reliant on transport of viable virus or whole, unprocessed antigen to organised lymphoid tissue by infected cells or by other antigen delivery processes. Marginal zone B cells in the spleen are able to take up blood-borne antigens, these cells constantly shuttle between the marginal zone and the follicle, carrying antigen to the FDCs (Cinamon *et al.*, 2008, Kraal, 2008). However, lymph nodes lack an equivalent B cell subset. Recent studies using two-photon intravital microscopy to visualise living cells deep within tissue of mice, have revealed a number of mechanisms of intact antigen delivery in lymph nodes. Soluble antigen from the periphery enters the subcapsular sinus of the draining lymph node via afferent lymphatic vessels. Studies in mice have shown that soluble antigen can diffuse across small gaps in the floor of the subcapsular sinus directly to nearby

B-cell follicles (Pape *et al.*, 2007). Following subcutaneous injection of antigen in mice, B cells in the follicle have been shown to take up antigen within 10 minutes, highlighting the rapid acquisition of soluble antigen by follicular B cells. These investigators also showed that by 4 hours post-inoculation, the antigen had already been processed and presented by B cells (Pape *et al.*, 2007) and that DCs do not play a major role in the acquisition of soluble antigen by follicular B cells (Cahalan and Parker, 2008). Recently, it has also been shown that soluble antigen can diffuse along a system of follicular conduits that connect the subcapsular sinus with the FDC areas, providing an alternative route for small lymph-borne antigens to the B-cell follicle (Roozendaal *et al.*, 2009). Subcapsular sinus macrophages are also able to transport antigen into the lymph node follicles (Martinez-Pomares *et al.*, 1996). B cells pick up the antigen displayed on the surface of these macrophages and transport the antigen to the follicle where the antigen is off-loaded onto FDCs (Phan *et al.*, 2007). These macrophages have been shown to clear lymph-borne VSV particles in mice and present the intact virion to B cells (Junt *et al.*, 2007). These investigators showed that splenic marginal-zone macrophages require complement and natural antibodies to capture live VSV, by contrast, the lymph-node resident macrophages retain VSV by means of complement and antibody independent mechanisms. It has been proposed that the virus is recognised by a scavenger receptor expressed by murine subcapsular sinus macrophages, for example, carbohydrate-binding scavenger receptors, but the specific receptor has not been identified (Taylor *et al.*, 2005). In addition, unlike other macrophages, the subcapsular sinus macrophages of mice are not highly phagocytic and do not rapidly degrade but retain surface-bound antigen (Cahalan and Parker, 2008, Phan *et al.*, 2007). DCs can also display unprocessed antigen on their

surface to B cells (Qi *et al.*, 2006). However, it has been shown in the mouse that DCs do not display whole antigen to follicular B cells and the B cells that recognise the surface antigen remain extrafollicular where they interact with T cells and do not enter the germinal centre reaction, although this process may be altered in the presence of live virus (Qi *et al.*, 2006). It is not implausible that different antigen delivery and antigen acquisition mechanisms in the different lymphoid tissues sampled during the current study are responsible for the observed distribution of FMDV capsid protein in the tissue sections. Intact capsid was detected in the palatine tonsils, lateral retropharyngeal lymph nodes and mandibular lymph nodes but not in dorsal soft palate and pharyngeal tonsil samples. In contrast to the lymph nodes, the organised lymphoid tissue within the mucosa of the dorsal soft palate and the pharyngeal tonsils are largely dependent on M cells for uptake of antigen from the lumen and the palatine tonsils are dependent on the crypt epithelium for antigen uptake (Kraehenbuhl and Neutra, 2000). In addition, the differences in antigen acquisition could also account for the low quantity of FMDV genome detected in splenic germinal centres, which was unexpected as FMDV infection in cattle generally results in a pronounced viraemia, and presumably widespread distribution of viral genome.

FMDV infection is characterised by a rapid and efficient isotype-class-switched neutralising-antibody response directed against viral B-cell epitopes. The viraemia is controlled rapidly and the animals soon recover from clinical FMD. However, low titres of live virus can still be recovered from oropharyngeal scrapings for months after infection despite the high titres of virus neutralising antibody and the prolonged

duration of immunity after natural infection. During the studies reported in this thesis, FMDV particles were detected up to 38 days post-infection in the light zone of germinal centres. Retention of intact FMDV particles on the FDC network provides an ideal mechanism for maintaining a highly cytopathic and lytic virus like FMDV extracellularly in a non-replicating, native, stable non-degraded state and may represent the reservoir of virus detected in 'carrier' animals. FDCs are able to maintain intact antigen beyond the contraction phase of the germinal centre response, a function that may be particularly relevant for infectious virus (Tew *et al.*, 1979). In addition, immune complexed FMDV can bind and infect Fc receptor expressing cells *in vitro*, potentially supporting an intermittent virus replication cycle in cattle in the presence of high titres of neutralising antibodies (Mason *et al.*, 1993, Rigden *et al.*, 2002, Robinson, 2008). We propose that viable virus detected in probang samples is due to direct harvesting of FDC-bound FMDV or as a consequence of virus originating from the FDC network and undergoing cycles of replication in susceptible cells, for example, macrophages, DCs or B cells, which will ensure efficient perpetuation of the virus within the host. Progeny virus produced by these cells could also infect other susceptible cells, for example, $\alpha\beta6$ expressing crypt epithelium cells. B cells could be particularly relevant for this model of FMD in cattle and their interactions with FMDV at different stages of development should be investigated. FDC-derived iccosomes are dispersed to B cells within the germinal centre, which endocytose and process the immune-complexed antigen (Tew *et al.*, 2001). In addition, naïve B cells constantly recirculate through the spleen, different lymph nodes and multiple germinal centres, scanning the antigen trapped on FDCs (Schwickert *et al.*, 2007). Recently, two-photon immunoinaging studies in mice

have shown that naïve B cells which enter the lymph node follow the scaffold of fibroblastic reticular cells until they reach the follicle, once within the follicle the B cells migrate along the scaffold formed by FDCs (Cahalan and Parker, 2008). This active process of scanning ensures that a large portion of the B-cell repertoire is exposed to antigen trapped in germinal centres, and any antigen-specific B cells will interact with the antigen (Schwickert *et al.*, 2007). Recently, human peripheral memory B cells that are latently infected with Epstein-Barr virus, have been shown to originate from germinal centres, for example tonsillar germinal centres, where the latent infection is established and rare persistently infected cells can be detected (Roughan and Thorley-Lawson, 2009). Therefore, pathogens residing in germinal centres can be actively transported to peripheral mucosal sites (Pegtel *et al.*, 2004).

Retention of non-degraded FMDV capsid in the light zone of germinal centres may also contribute to the generation of long-lasting neutralising-antibody responses either as a direct result of persisting viral antigen or the production of memory cell populations and long lived plasma cells. FMDV is highly immunogenic and FMDV infection is able to induce a rapid and specific T-I virus neutralising antibody response in cattle. Therefore, the rapid evolution of antigenic sites and the diverse genetic and antigenic heterogeneity of FMDV are likely to be biologically relevant for virus survival at a population level in order to circumvent detection and destruction in the face of an effective host immune response (Domingo *et al.*, 2003). Consequently, it would seem that a balance has been reached between the bovine immune system and the virus to guarantee survival of both the virus and the host (Zinkernagel, 1996).

The proposed model of host-pathogen interaction may be particularly relevant to understand how FMDV persists and spreads in the major wildlife reservoir of FMDV in Sub-Saharan Africa, the African buffalo (*Syncerus caffer*). Free-living African buffalo act as maintenance host for the three SAT serotypes of FMDV in southern Africa and have been shown to be a source of infection for other susceptible species with transmission from ‘carrier’ buffalo to cattle reported under experimental conditions (Vosloo *et al.*, 2002). This is in contrast to the unknown epidemiological significance of ‘carrier’ cattle. Transmission from ‘carrier’ cattle has not been demonstrated under experimental conditions, even during dexamethasone treatment and under conditions of co-infection with other viruses, for example, rinderpest and bovine herpes 1 viruses (Ilott *et al.*, 1997, McVicar, 1977). Epidemiological data indicates that in areas where SAT serotypes are prevalent, for example, the Kruger National Park in South Africa, buffalo are infected with all three SAT types by 2 years of age (Thomson *et al.*, 1992). The SAT viruses produce cyclical epidemics of infection in young buffalo within breeding herds when susceptible calves, whose maternal antibody has waned, are recruited into the population. The virus is subsequently transmitted to other susceptible species (usually cattle or impala) with which these breeding herds come into contact (Thomson *et al.*, 1992). FMDV has been successfully isolated from captive buffalo held in isolation for 5 years and from a small, free-living isolated population for 24 years (Condy *et al.*, 1985). Within breeding herds, the virus only needs to persist in immune animals between calving seasons for transmission to the next generation of susceptible calves by a method that remains to be elucidated.

Although these data support a novel reservoir of FMDV, viable virus was not isolated from lymphoid tissue. In addition, these data provide no evidence to support Fc receptor mediated virus replication *in vivo* and the sites of replicating virus in cattle after recovery from FMDV have not been determined. Buffalo harbour persistent virus in greater amounts and for longer periods than cattle, therefore they provide a better opportunity to define the sites of virus localisation, to progress studies to isolate live virus from these sites and to elucidate the mechanism whereby virus is transmitted from 'carrier' to susceptible buffalo. The inability to detect live FMDV in bovine lymphoid tissue samples is likely due to technical difficulties extracting virus from bovine tissue. FDCs remain a challenging cell type to study, especially in cattle. Therefore, in addition to studying the virus in buffalo, the murine model system could be used to attempt to detect FMDV protein and genome in germinal centres. In addition, established protocols for detecting viable HIV on mouse FDCs could be followed in an attempt to detect viable FMDV (Smith *et al.*, 2001).

Despite the uncertainty of the requirement of persisting antigen to maintain humoral immunity, it is clear that serum antibodies have a short half-life and require replenishment either by long-lived plasma cells, activation of memory B cells to differentiate into plasma cells or by the ongoing recruitment and differentiation of naïve B cells into antibody producing cells. It is anticipated that FMDV maintained on FDCs in the light zone of germinal centres plays a crucial role in maintaining humoral immunity. In addition, in FMD convalescent cattle it has been shown that resistance to re-infection and local virus replication in the oropharynx shows a strong

correlation with a history of persistent infection (McVicar and Suttmoller, 1974, Salt, 1993). The elimination of sequestered antigen on FDCs by injection of LT β R-Ig fusion proteins during the early stages of the germinal centre reaction has been shown to have a detrimental effect on antibody titres in mice, highlighting the importance of persisting antigen during the early phase of the B-cell response when germinal centres are producing large numbers of plasma and memory B-cell precursors (Gatto *et al.*, 2007). Although it is not feasible to replicate these studies in cattle at this stage, replicating these studies using the FMDV murine model system will provide data on the importance of FMDV retention, in early and late germinal centres, for maintaining SNTs.

It is interesting to note that pigs are reported to clear FMDV within 3 to 4 weeks post-infection. In addition, FMDV infection in pigs induces neutralising titres of antibody that are only detectable for a few months post-infection, with a reported half-life of 1 week (Alexandersen *et al.*, 2003b). This is an unusual host response to a highly immunogenic and cytopathic virus like FMDV (Hangartner *et al.*, 2006, Manz *et al.*, 2005), sanctioning further investigation in order to understand the germinal centre reaction and localisation of virus in pigs following FMDV infection.

The results reported in this thesis support the hypothesis that the rapid induction of protective neutralising antibody following natural FMDV infection in cattle is dependent on the highly repetitive and ordered structure of the FMDV capsid. In addition, the long-term maintenance of protective SNTs following infection is likely to be dependent on non-degraded FMDV persisting in germinal centres. The

induction of rapid and long-lasting protective immunity is the primary aim for successful vaccination against infectious diseases. Clearly, FMDV infection in cattle induces robust, long lasting immunity and vaccine-induced immunity which mimics natural infection should induce a similar degree of protection. Immunisation with a single dose of commercial, inactivated FMDV vaccine can induce rapid SNTs in ruminants, protection from clinical disease and prevent virus dissemination (Cox *et al.*, 1999, Cox *et al.*, 2005). However, vaccination and even in some cases previous infection, does not always confer protection against local virus replication or superinfection and the potential for transmission. In FMD convalescent cattle, resistance to local virus replication in the oropharynx correlates with a history of persistent infection (McVicar and Suttmoller, 1974, Salt, 1993, Salt *et al.*, 1996a). Antibody in the upper respiratory tract and oral secretions are reported to persist for longer and at a higher titre in ‘carrier’ compared to ‘non-carrier’ animals (Garland, 1974, Matsumoto *et al.*, 1978). In addition, IgA titres persist in serum and in probang samples of ‘carrier’ animals (Salt *et al.*, 1996a) and it has been proposed that neutralising IgA in secretory fluids of these animals is primarily responsible for preventing local FMDV replication in the mucosa (Garland, 1974, Matsumoto *et al.*, 1978). The role of IgA versus IgG in the control on FMDV in the upper respiratory tract is not clear. The predominant antibody isotypes detected in probang samples following inoculation of vaccine intramuscularly, and even when administered into the muzzle of cattle, are IgG1 and IgG2 and low titres of IgA only become detectable after multiple re-vaccinations (Archetti *et al.*, 1995, Barnett *et al.*, 1998). Studies in ruminants have shown that increasing the antigen payload in single dose emergency vaccines administered intramuscularly can prevent or decrease local virus replication

and prevent persistence and shedding, suggesting that a robust systemic antibody response induced by the inactivated virus capsid is adequate for protection at the mucosal surface in the absence of local IgA, as determined by probang sampling (Barnett *et al.*, 2004, Cox *et al.*, 2006). Mucosal plasma cells produce IgG, however, the majority of IgG at the mucosal surfaces is derived from the plasma by a process of passive transudation along a concentration gradient (Lamm, 1997). In addition, any local inflammation or damage during FMDV infection will allow the IgG to diffuse across the epithelium but only after the virus has initiated infection.

Therefore, it is likely that the majority of IgG detected in probang samples following intramuscular vaccination is not locally produced but serum derived and that high SNTs are required if local virus replication and shedding at the mucosal surface is to be prevented (Barnett *et al.*, 1998, Wagner *et al.*, 1987). Passive immunisation studies in mice have shown that IgA, but not IgG, can prevent influenza virus induced pathology in the upper respiratory tract (Renegar *et al.*, 2004). In addition, titres of circulating IgG 2.5 times the normal convalescent serum anti-influenza virus titre was required for antibody transudation into nasal secretions and 7 times normal was required to lower nasal virus shedding by 98%. These authors concluded that IgG did not prevent the initiation of viral infection at the mucosa but neutralised locally produced virus (Renegar *et al.*, 2004).

The intact virus particle, which is the major immunogenic component of current FMD vaccines, is adversely affected by aziridine compounds during inactivation. The subsequent thermal instability and spontaneous dissociation of the capsid means that the highly repetitive and ordered conformational epitopes are not well preserved,

altering the immunogenic properties of the virus particle (Anderson *et al.*, 1983, Bahnmann, 1975, Doel and Baccarini, 1981, Patil *et al.*, 2002). In addition to the highly repetitive and ordered structure, live virus also induces innate immunity for appropriate conditioning of adaptive immune responses and the ability to replicate leads to virus distribution promoting efficient B and T-cell responses for robust and long-lived immunity (Jennings and Bachmann, 2008). Increasing the antigen payload of inactivated vaccines can overcome some of their limitation and it should be noted that to date, there have been no published experiments in either cattle or sheep immunised with a single dose of higher potency vaccine and then challenged at a time point beyond 28 post-vaccination to assess long-term protection. Current efforts focussed on the production of stable virus capsids in order to preserve conformational epitopes are promising for providing the next-generation of FMDV vaccines with the goal of being safer to produce and to trigger rapid and sustained antibody responses. Once synthesised, the empty capsids can form a platform for vaccine technology based on virus-like particles (VLPs) (Jennings and Bachmann, 2008). During the past 20 years, VLP-based vaccines have been the subject of extensive research and currently VLPs derived from human papillomavirus and hepatitis B virus are marketed for human use (Barr and Tamms, 2007, Jennings and Bachmann, 2008). VLPs mimic natural virus infection to trigger an effective immune response, their particulate nature and size means they are effectively transported to lymph nodes to display ordered and repetitive antigen to follicular B cells (Jennings and Bachmann, 2007). In addition, VLPs which spontaneously assemble, for example, the L1 major capsid protein of papillomaviruses, can be packaged with TLR ligands and other activators of innate immunity in order to direct the subsequent

adaptive immune response (Jennings and Bachmann, 2008). Although VLPs have been shown to induce adequate B-cell responses in mice in the absence of adjuvant (Gatto *et al.*, 2004), incorporating depot forming adjuvants may extend the duration of immunity by replicating persisting antigen on FDCs after natural infection. The quadrivalent human papillomavirus VLP vaccine which incorporates an alum-based adjuvant has been shown to induce antibody titres in humans that peak at 7 months then decrease to a plateau titre that is maintained for 5 years after a single dose (Olsson *et al.*, 2007). However, diffusion of soluble antigen away from adjuvant depots is limited and it has been reported that antigen delivery from adjuvant depots to lymph nodes in mice is primarily by DCs, which may ultimately reduce the amount of intact antigen reaching the lymph node and the amount of antigen entering the germinal centre reaction (Cahalan and Parker, 2008). Therefore, depots of FMDV capsid at peripheral vaccination sites in cattle may not functionally replicate the depots of intact virus after natural infection which is maintained on FDCs in direct contact with follicular B cells.

In conclusion, the data presented here provides fresh insight into the induction and maintenance of the protective immune response against FMDV in the natural bovine host. Many issues which are pertinent to understanding the protective immune response and the 'carrier state' remain to be elucidated. However, extrapolating the data reported here with the data provided by two-photon microscopy of host-pathogen interactions *in vivo* can inform FMDV vaccine strategies which attempt to mimic the immunogenicity of natural infection.

5. References

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Appendix 1: medium, buffers and solutions

Acetylation solution

The solution was prepared immediately before use.

49ml nuclease free water (Ambion, UK)

660µl triethanolamine solution (Ambion, UK)

250µl HCl 37% (Sigma-Aldrich, UIK)

60µl acetic anhydride \geq 98% pure (Ambion, UK)

Agarose gel (1%)

1 \times TBE electrophoresis buffer

- 89mM Trizma-base (Sigma-Aldrich, UK)
- 89mM boric acid (Sigma-Aldrich, UK)
- 2mM ethylenediaminetetraacetic acid disodium salt dehydrate (Sigma-Aldrich, UK)
- distilled water (CSU, IAH)

1% (w/v) agarose (Promega, UK)

0.002% of 10mg/mL ethidium bromide (Sigma-Aldrich, UK)

Cell culture medium

Baby hamster kidney (BHK-21) cells

- Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, UK)
- 10% v/v fetal calf serum (Autogen Bioclear, UK)
- 20mM glutamine (CSU, IAH)
- 100µg/mL streptomycin (CSU, IAH)
- 100 SI units/mL penicillin (CSU, IAH)
- Incubated at 37°C, 5% CO₂

Primary bovine thyroid (BTY) cells

- Glasgows Modified Eagle's Medium (GMEM, CSU, IAH)
- 10% v/v fetal calf serum (Autogen Bioclear, UK)
- 100µg/mL streptomycin (CSU, IAH)
- 100 SI units/mL penicillin (CSU, IAH)
- Incubated at 37°C, 5% CO₂

Mouse fibroblast 3T3 cells

- DMEM (Sigma-Aldrich, UK)
- 10% v/v fetal calf serum (Autogen Bioclear, UK)
- 100µg/mL streptomycin (CSU, IAH)
- 100 SI units/mL penicillin (CSU, IAH)
- Incubated at 37°C, 5% CO₂

Colour substrate solution

5ml detection buffer

25µl of 100mg/ml nitroblue tetrazolium chloride (Roche, UK)

18.75µl of 50mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (Roche, UK)

1mM levamisol (Sigma-Aldrich, UK)

Detection buffer

0.1M Trizma-base (Sigma-Aldrich, UK)

0.1M sodium chloride (Sigma-Aldrich, UK)

pH 9.5 hydrochloric acid (Sigma-Aldrich, UK)

FACS wash buffer

PBS (CSU, IAH)

1% (w/v) bovine serum albumin (Sigma-Aldrich, UK)

3mM sodium azide (Sigma-Aldrich, UK)

Filter sterilise

Hybridization buffer

40% (v/v) deionised formamide (Sigma-Aldrich, UK)

10% (v/v) dextran sulfate (Sigma-Aldrich, UK)

1× Denhardt's solution (Sigma-Aldrich, UK)

4×SSC buffer (Sigma-Aldrich, UK)

10mM dithiothreitol (Sigma-Aldrich, UK)

1mg/ml yeast t-RNA (Roche, UK)

1mg/ml denatured and sheared salmon sperm DNA (Roche, UK).

The prepared buffer was replaced with the hybridization buffer supplied in the mRNA Locator *in situ* Hybridization Kits (Ambion, UK). The buffers in this kit are optimised for use with radiolabelled RNA probes. DIG labelled probes and ³³P labelled probes behave with similar kinetics and may be used under similar hybridization conditions (Sambrook and Russel, 2001).

Luria-Bertani agar (CSU, IAH)

Luria-Bertani broth with 1.5% (w/v) agar

Luria-Bertani broth (CSU, IAH)

1% (w/v) tryptone

0.5% (w/v) yeast extract

0.5% (w/v) sodium chloride

Distilled water

pH 7.6 Trizma-base

M25-phosphate buffer, pH 7.6 (CSU, IAH)

35 mM disodium hydrogen orthophosphate dihydrate

5.7 mM potassium dihydrogen phosphate

Pre-hybridization buffer

Initially a solution of prepared hybridization buffer without dextran sulfate was used for pre-hybridization. This was replaced by the pre-hybridization buffer supplied in the mRNA Locator *in situ* Hybridization Kits (Ambion, UK).

Red blood cell lyses buffer

0.115M ammonium chloride (Sigma-Aldrich, UK)
1mM potassium hydrogen carbonate (Sigma-Aldrich, UK)
0.01mM ethylenediaminetetraacetic acid disodium salt dehydrate (Sigma-Aldrich, UK)
pH 7.2 (1M sodium hydroxide [Sigma-Aldrich, UK])
0.22µm filter sterilise (Sartorius, UK)

RNA digestion solution

0.001 µg/mL RNase A (Ambion, UK)
1 × RNase digestion buffer (Ambion, UK)
Distilled water

SOC media (CSU, IAH)

2% (w/v) tryptone
0.5% (w/v) yeast extract
10mM sodium chloride
2.5mM potassium chloride
20mM magnesium chloride
20mM D(+) glucose
Autoclaved to sterilise

TaqMan Reverse Transcription Reagent reaction mix (Applied Biosystems, UK)

1 × TaqMan RT buffer
5.5mM magnesium chloride
500µM deoxyNTPs mixture
2.5µM random hexamers
0.4U/µL RNase inhibitor
1.25U/µL Multiscribe reverse transcriptase
RNase-free water

TBS washing buffer

0.1M Trizma-base (Sigma-Aldrich, UK)
0.15M sodium chloride (Sigma-Aldrich, UK)
pH 7.5 hydrochloric acid (Sigma-Aldrich, UK)

TBST blocking buffer

0.1M Trizma-base (Sigma-Aldrich, UK)

0.15M sodium chloride (Sigma-Aldrich, UK)

0.1% (v/v) Tween 20 (Sigma-Aldrich, UK)

2% (v/v) normal sheep serum (Sigma-Aldrich, UK) for blocking, 1% (v/v) for incubation with antibody

pH 7.5 hydrochloric acid (Sigma-Aldrich, UK)

TNB buffer

0.1M Trizma-base (Sigma-Aldrich, UK)

0.15M sodium chloride (Sigma-Aldrich, UK)

0.5% (w/v) PerkinElmer TSA blocking reagent (PerkinElmer, UK)

pH 7.5 hydrochloric acid (Sigma-Aldrich, UK)

TNT buffer

0.1M Trizma-base (Sigma-Aldrich, UK)

0.15M sodium chloride (Sigma-Aldrich, UK)

0.3% (v/v) Triton X-100 (Sigma-Aldrich, UK)

pH 7.5 hydrochloric acid (Sigma-Aldrich, UK)

Appendix 2: Primers and probes

Primer or probe name/number	Accession number	Probe and primer sequence (5' to 3')
28sF	AY639443	20-(GCG GAA TTC)-CGG TCC TGA CGT GCA AAT-37
28sR	AY639443	280-(GCG GGA TCC)-CTA GGC ACT CGC ATT CCA C-262
SA-UK-IRES-308R	AJ539141	983-CCG AGT GTC GCG TGT TAC CT-964
SA-UK-IRES-248F	AJ539141	923-AAC CAC TGG TGA CAG GCT AAG G-944
UK-IRES-271T	AJ539141	946-TGC CCT TTA GGT ACC C-961
SA-IR-219-246F	AJ539141	893-CAC YTY AAG RTG ACA YTG RTA CTG GTA C-920
SA-IR-315-293R	AJ539141	989-CAG ATY CCR AGT GWC ICI TGT TA-967
SAmulti2-P-IR-292-269R	AJ539141	949-CCT CGG GGT ACC TGA AGG GCA TCC-972
Callahan 3DF	AJ539141	7863-ACT GGG TTT TAC AAA CCT GTG A-7884
Callahan 3DR	AJ539141	7969-GCG AGT CCT GCC ACG GA-7953
Callahan 3DP	AJ539141	7914-TCC TTT GCA CGC CGT GGG AC-7933
P15 Poly A	AJ539141	GGC GGC CGC TTT TTT TTT TTT TTT
FMDV 1F	AJ539141	6678-(GCGGAA TTC)-GGA TTG ATA GTT GAC ACC AGA GAT G-6702
FMDV 1R	AJ539141	7177-(GCG GGC TCC)-ATC TCG TCC TTC AGG AAG GTC-7157
IgG1F	S82409	305-(GTT GCG GCC GCA A)-GC AAA ACA ACC TGT GAC TGT TG-326
IgG1R	S82409	990-(ACG TGG AAT)-TCA TTT ACC CGC AGA CTT AGA GG-968
CD321F	BC113215	10-AAG AAG CCA GTG CCT GTC GT-30
CD321R	BC113215	1058-CTG CAG TAT CCT CTT CTA CCC AA-1035
CD32Fmutant	BC113215	798-CCT GGT TCC GCC TCA GGT GAT AGT CAC TCT CAG CCG GTC TCA C-840
CD32Rmutant	BC113215	840-GTG AGA CCG GCT GAG AGT GAC TAT CAC CTG AGG CGG AAC CAG G-798

Appendix 3: List of publications

Sections of this thesis have been published in the following scientific publications:

Juleff, N., Windsor, M., Reid, E., Seago, J., Zhang, Z., Monaghan, P., Morrison, I. W. & Charleston, B. (2008) Foot-and-mouth disease virus persists in the light zone of germinal centres. *PLoS ONE*, 3, e3434.

Juleff, N., Windsor, M., Lefevre, E. A., Gubbins, S., Hamblin, P., Reid, E., McLaughlin, K., Beverley, P. C., Morrison, I. W. & Charleston, B. (2009) Foot-and-mouth disease virus can induce a specific and rapid CD4⁺ T-cell-independent neutralising and isotype class switched antibody response in naive cattle. *Journal of Virology*, 83, 3626-3636.

Additional publications:

Cottam, E. M., Wadsworth, J., Shaw, A. E., Rowlands, R. J., Goatley, L., Maan, S., Maan, N. S., Mertens, P. P., Ebert, K., Li, Y., Ryan, E. D., Juleff, N., Ferris, N. P., Wilesmith, J. W., Haydon, D. T., King, D. P., Paton, D. J. & Knowles, N. J. (2008) Transmission pathways of foot-and-mouth disease virus in the United Kingdom in 2007. *PLoS Pathogens*, 4, e1000050.

Ryan, E., Gloster, J., Reid, S. M., Li, Y., Ferris, N. P., Waters, R., Juleff, N., Charleston, B., Bankowski, B., Gubbins, S., Wilesmith, J. W., King, D. P. & Paton, D. J. (2008) Clinical and laboratory investigations of the outbreaks of foot-and-mouth disease in southern England in 2007. *Veterinary Record*, 163, 139-47.